FABP4: INTERACTIONS WITH ENDOTHELIAL CELL PLASMA MEMBRANE AND EFFECTS ON VASCULAR SMOOTH MUSCLE CELLS.

Paula Saavedra García

Dipòsit Legal: T 238-2016

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FABP4: INTERACTIONS WITH ENDOTHELIAL CELL PLASMA MEMBRANE AND EFFECTS ON VASCULAR SMOOTH MUSCLE CELLS

PAULA SAAVEDRA GARCÍA

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We STATE that the present study, entitled "**FABP4: Interactions with endothelial cell plasma membrane and effects on vascular smooth muscle cells**", presented by **Paula Saavedra García** for the award of the degree of Doctor, has been carried out under my supervision at the Department of Medicine and Surgery of this university.

Reus, 3rd November 2015

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Learn from yesterday, live for today, hope for tomorrow.

The important thing is not to stop questioning.

Albert Einstein

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Agradecimientos

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Halloween con vampiru incluyíu, por Vielha, pol *drinking game*, polos *kalitxikis*, por los *¡arriba!*, pol caballu y la peonza, poles cañes de los xueves, poles *cena+copa+baille*, polos vermús electrónicos (y polos non electrónicos tamién), por Catalina y Kochinova, poles paelles na sablera, poles visites, por tener sío la mio familia y por cuidame tanto.

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The project of the thesis I present has been developed in the Lipids and Atherosclerosis Research Unit (URLA) that belongs to the Department of Medicine and Surgery in the Faculty of Medicine and Health Sciences at the Rovira i Virgili University and has been directed by Prof. Lluís Masana and Dr. Josefa Girona.

The main objective of this research unit, on a broad scale, is to contribute with the cardiovascular morbidity and mortality reduction through the study of the lipid metabolism, the mechanisms involved in atherosclerosis, their clinical manifestations and risk factors. Cardiovascular diseases were the leading cause of non-communicable disease deaths in 2012 and were responsible for 17.5 million deaths. The pathophysiological basis of most of the cardiovascular diseases is the atherosclerosis that is the leading cause of death in the developed world. In the last years much effort has been done to improve its pharmacological therapy. In this study, we have tried to contribute with the understanding of Fatty acid-binding protein 4 (FABP4) and its interaction with endothelium. It is known that there are links between plasma levels of FABP4 and different cardiovascular pathologies, however, the mechanism of action of circulating FABP4 is not clear. This thesis is composed by two different studies, one done in vascular endothelial cells and the other one in vascular smooth muscle cells. The results of these two studies give us a global idea of how circulating FABP4 affects the endothelium.

This thesis is divided into different sections. The **Introduction** provides the general knowledge necessary to understand the course of work. Then, **Hypothesis and objectives** section which justifies the development of experimental studies. The **Material and methods** and **Results** of the thesis are described in three manuscripts, two of which are published in international scientific journals (*BBA Molecular Cell Research* and *PLOS ONE*) and are included in the main text of the thesis. The third one is published in the journal *Clínica e Investigación en*

Arteriosclerosis (**Annex I**). The **Summary results** section abstracts the main results of the papers. After, the **Discussion** and the **Conclusions** of the thesis and finally, all **References** used in this work.

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2. Introduction

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2.1. Cardiovascular diseases and associated disorders

Cardiovascular diseases (CVDs) were the leading cause of non-communicable disease deaths in 2012 and were responsible for 17.5 million deaths, which was 46% of non-communicable disease deaths. Of these deaths, an estimated 7.4 million were due to heart attacks (ischemic heart disease), and 6.7 million were due to stroke[1, 2]. Predisposing CVD risk factors, such as increased adiposity and body fat distribution, physical inactivity, and poor diet, may modulate CVD risk in part through overlapping effects on hypertension, dyslipidaemia, and type 2 diabetes mellitus (T2DM) itself and possibly via other mechanisms[3]. The gains in CVD prevention are now seriously challenged by the impact of global epidemics of obesity, metabolic syndrome (MS), and T2DM[4]. Current standards of care for primary and secondary CVD prevention emphasize the importance of multifactorial intervention to achieve recommended targets for low-density lipoprotein (LDL) cholesterol, blood pressure, and glycaemic control[5].

Obesity is closely associated with an increased risk for a cluster of metabolic and CVDs[6]. Abundant evidence has demonstrated that obesity is a state of low-grade chronic inflammation that triggers the release of lipids, aberrant adipokines, pro-inflammatory cytokines, and several chemokines from adipose tissue. This low-grade inflammation, caused by overnutrition, is a major cause of decreased insulin sensitivity and underlies the development of insulin resistance (IR) and associated metabolic comorbidities, such as T2DM and non-alcoholic fatty liver disease (NAFLD)[7]. NAFLD is strongly associated with obesity, IR and T2DM, but many people with NAFLD are not obese and do not have T2DM[8]. IR is a complicated condition in which three primary metabolic tissues that are sensitive to insulin, skeletal muscle, liver, and white adipose tissue become less sensitive to insulin and its downstream metabolic actions under normal serum glucose concentrations[9].

T2DM has reached epidemic proportions around the world, and the increase in cardiovascular risk attributable to T2DM, estimated to range from 2- to 4-fold, poses a serious public health concern[3]; in fact, cardiovascular complications are the most common and devastating complications of T2DM. They are a major cause of hospital admissions and the leading cause of death among diabetic patients. A prominent attribute of diabetic cardiovascular complications is accelerated atherosclerosis, which is associated with oxidative stress, IR, and MS[10]. MS is defined as a set of CVD and T2DM risk factors occurring together rather than separately, including abdominal obesity, increased fasting plasma glucose, hypertension, and dyslipidemia[11]. MS should be considered a clinical entity when its different symptoms share a common aetiology: obesity/IR as a result of multi-organ dysfunction and its components drastically increases the risk of atherosclerosis[12]. Atherosclerosis is the pathophysiological basis of all of these CVDs.

2.2. Atherosclerosis

Atherosclerosis is a disease of the arterial wall that occurs at susceptible sites in the major conduit arteries. Lesions begin in the inner lining of the arteries—the intimae—and progressively affect the entire arterial wall, including the media and adventitia[13]. It is a chronic inflammatory condition of the vessel wall that results from the transendothelial passage (transcytosis) of cholesterol-rich atherogenic apolipoprotein (Apo)-B lipoproteins (very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL) and LDL) from the plasma into the intima[14, 15]. These lipoproteins are retained in the subendothelial space, which leads to infiltration of macrophages and T cells that then ultimately interact with each other

and with the cells of the arterial wall[14]. Lipoproteins trigger a non-resolving inflammatory response in the various lesional cell types, and it is the inflammatory cells in the lesions that trigger the type of plaque changes that lead to CVD[16].

Several risk factors may intensify or provoke atherosclerosis through their effects on LDL particles and inflammation. These risk factors most frequently include hypertension, tobacco smoking, T2DM, obesity, and genetic predisposition[13]. Risk factors play an important role in initiating and accelerating the complex process of atherosclerosis and are also the primary method of risk assessment and the target for therapeutic intervention in the prevention of premature vascular disease[17].

2.2.1. Pathogenesis of atherosclerosis

The vascular network is composed of differently sized vessels, including micro, small, medium, and large vessels. Vascular tissues include several types of cells, including endothelial cells, vascular smooth muscle cells (VSMCs), pericytes, fibroblasts, resident macrophages, resident mesenchymal stem cells and progenitors, and connective tissues. The classical three-layer structure of the vascular wall involves the intima, media, and adventitia flanked with the elastic laminae (Fig. 1)[15]. These endothelial cells are characterized by their stratification and are connected via intercellular junctions that confer specific permeability. The vascular endothelium is divided into arterial and venous endothelia with additional differences between larger and smaller vessels[18, 19].

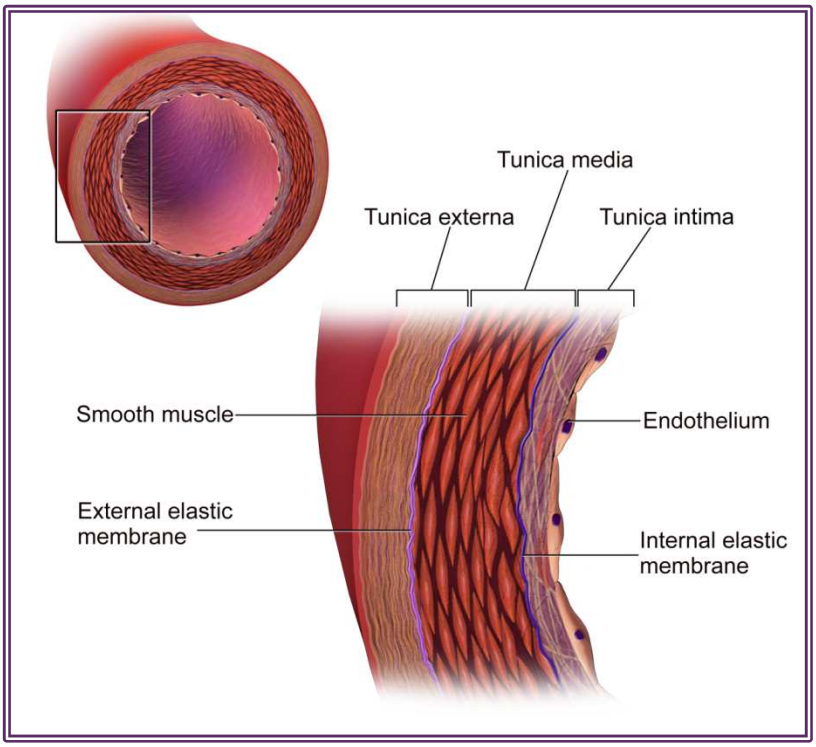

Figure 1. Structure of an artery wall (Blausen.com staff. "Blausen gallery 2014". Wikiversity Journal of Medicine. DOI:10.15347/wjm/2014.010. ISSN 20018762).

Atherogenesis starts with an initial qualitative change in the monolayer of endothelial cells that lines the inner arterial surface (Fig. 2A). Arterial endothelial cells express adhesion molecules that capture leukocytes on their surfaces (Fig. 2B). Parallel changes in endothelial permeability and the composition of the extracellular matrix beneath the endothelium promote the entry and retention of cholesterol-containing LDL particles in the artery wall[20]. Chemoattractant mediators direct the migration of the leukocytes into the tunica intima (Fig. 2B), where monocytes differentiate into tissue macrophages. In the nascent atheroma, these macrophages engulf lipoprotein particles and become foam cells[21].

Atheroma formation also involves the recruitment of VSMCs from the tunica media into the tunica intima (Fig. 2C), which contains resident VSMCs. During atherogenesis, other VSMCs migrate from the media into the intima and proliferate in response to mediators, such as platelet-derived growth factor (PDGF). In the intima, the VSMCs produce extracellular matrix molecules, including interstitial collagen and elastin, and form a fibrous cap that covers the plaque. This cap typically overlies a collection of macrophage-derived foam cells, some of which die and release lipids that accumulate extracellularly. The inefficient clearance of dead cells, a process known as efferocytosis, can promote the accumulation of cellular debris and extracellular lipids, forming a lipid-rich pool called the necrotic core of the plaque[21, 22].

Plaques generally cause clinical manifestations by producing flow-limiting stenosis that leads to tissue ischemia or by provoking thrombi that can interrupt blood flow. Thrombi often arise after physical disruption of the plaque, most commonly a fracture of the fibrous cap that exposes procoagulant material in the plaque's core to coagulation proteins in the blood, triggering thrombosis (Fig. 2D)[21].

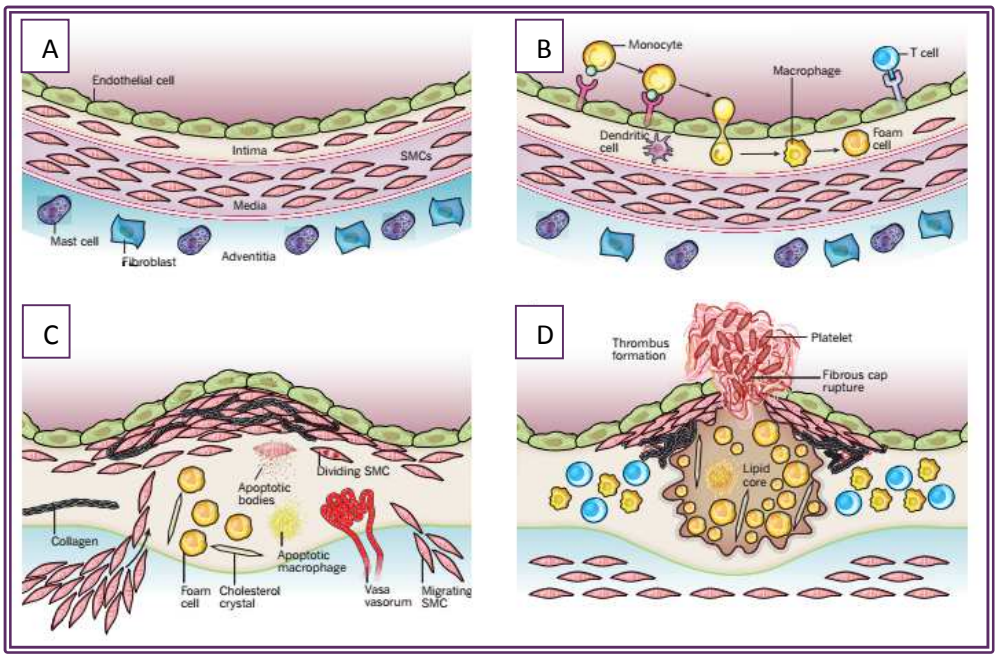

Figure 2. Stages in the development of atherosclerotic lesions[21].

Atherosclerosis involves several highly interrelated processes, including endothelial dysfunction, vascular smooth cell migration and proliferation, lipid disturbances, platelet activation, thrombosis, inflammation, oxidative stress, altered matrix metabolism, remodelling, and genetic factors[17].

2.2.2. Endothelial dysfunction

Endothelial dysfunction represents a switch from a quiescent phenotype toward one that involves the host defence response[23] and is characterized by a shift in the actions of the endothelium toward reduced vasodilation, a pro-inflammatory state, and prothrombic properties[24]. Apart from atherosclerosis, endothelial dysfunction

is also associated with most forms of CVD, such as hypertension, chronic heart failure, peripheral vascular disease, T2DM, chronic kidney failure, and severe viral infections[24]. Endothelial dysfunction due to breakdown of the endothelial barrier promotes atherogenesis as a result of enhanced permeability through the endothelial layer, increased adherence of leukocytes, monocytes and macrophages, and subendothelial accumulation of cholesterol-bearing lipoproteins[25]. Furthermore, free radicals can disrupt the balance of nitric oxide (NO) and damage the endothelium, leaving it overly permeable and allowing toxins to pass into body tissues[24]. In endothelial cells, NO is the most important mediator[26, 27] and is essential for the maintenance of integrity and homeostasis of endothelium[19]; it was originally identified as endothelium-derived relaxing factor[28]. NO is generated from L-arginine by the action of endothelial NO synthase (eNOS) in the presence of cofactors such as tetrahydrobiopterin[23] and plays a key role in vasodilation, inflammation and oxidative stress, mainly through the production of reactive oxygen species (ROS). Diminished NO bioactivity may facilitate vascular inflammation that could lead to oxidation of lipoproteins and foam cell formation, the precursor of an atherosclerotic plaque[27]. In obesity, circulating mononuclear cells are activated and are also considered to play a role in the induction of endothelial dysfunction. Transmigration of blood monocytes into adipose tissue is a complex mechanism that requires expression of adherent molecules on both monocytes and the endothelial cell layer to which monocytes attach[29].

In the quiescent state of the endothelium, NO is generated by the endothelial isoform of eNOS in its membrane-bound configuration. The released NO targets cysteine groups in key regulator molecules and the mitochondria, which leads to silencing of cellular processes[23]. The importance of the endothelium was first recognized by its effect on vascular tone, which is achieved by the production and

release of several vasoactive molecules that relax or constrict the vessel[23]. Dilating agents include NO, prostacyclin, bradykinin, and endothelium-derived hyperpolarizing factor; constricting agents include endothelin, superoxide anion, endothelium-derived constricting factor that is poorly characterized, locally produced angiotensin II, and thromboxane[30].

In atherosclerosis, the function of eNOS is altered to produce superoxide instead of NO; endothelial dysfunction is characterized by a decrease in NO bioactivity with a concomitant increase in superoxide formation[31]. In addition, eNOS may be activated by signalling molecules such as bradykinin, adenosine, vascular endothelial growth factor (VEGF) (in response to hypoxia), and serotonin (released during platelet aggregation)[23]. Bradykinin is an endogenous non-apeptide that exerts multiple functions the in cardiovascular system. Physiologically, bradykinin exerts its actions through activation of B2 receptors, leading to NO release[32, 33]. It regulates human coronary vascular tone and contributes to the improvement of systemic and coronary endothelial dysfunction during angiotensin-converting enzyme (ACE) inhibition and to the cardioprotection of myocardial ischemia by preconditioning and by ACE inhibitors and angiotensin AT1 receptor blockers. Bradykinin delivery and vascular biology, together with stimulation of prostaglandin synthesis and NO formation, are modulated by cytokeratin 1 (CK1) in endothelial cells. CK1 is a kininogen-binding protein detected at the cell surface and appears to play a role as an anchor or receptor for various active molecules[34]. In addition, chronic bradykinin infusion prevents the progression of heart failure by preserving LV diastolic and systolic functions and vascular endothelial function, which is probably related to a preserved eNOS expression[33].

2.2.3. Vascular smooth muscle cell proliferation and migration

VSMCs retain remarkable plasticity during postnatal development and can undergo dedifferentiation to a synthetic phenotype. The normally quiescent VSMCs within the medial layer of the vessel wall are activated to migrate and proliferate in response to increased stimulatory growth factors and cytokines (PDGF, interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF- α)) and reduced endothelium-derived inhibitory factors (NO, heparin sulfate proteoglycan). Some of the neointimal smooth muscle cells may also originate from the adventitial fibroblasts, which migrate to the intima and differentiate in myofibroblasts[35].

In atherosclerosis, VSMCs contribute to vessel wall inflammation and lipoprotein retention, as well as to the formation of the fibrous cap that provides stability to the plaque[36]. The proliferation and directed migration of abnormal VSMCs from the media into the intima play major roles in the pathogenesis of atherosclerotic lesions, the occurrence of restenosis after angioplasty, and accelerated arteriopathy after cardiac transplantation[37]. VSMC migration is a normal process that occurs during vascular development or for tissue repair in response to vascular injury. The distinction between physiological and pathological migration has been attributed to a failure to cease migration once tissue repair has been completed[37]. Cell migration begins with the stimulation of cell surface receptors that transduce the external signal to a series of coordinated remodelling events that alter the structure of the cytoskeleton[38]. Early signalling events trigger actin polymerization, which causes protrusion of the leading edge of the cell toward a chemotactic stimulus or along a path of varying adhesiveness within the extracellular matrix. New focal contacts form just behind the leading edge to increase adhesion of the cell membrane to the matrix[38]. The actomyosin motor assembles in the cell body, providing a contraction that, along with cytoskeletal remodelling and detachment

of focal contacts at the trailing edge, propels the cell toward the stimulus. All organelles of the cell are carried along with the remodelling cytoskeleton, possibly by adaptor and motor proteins that tether them to the cytoskeleton[38]. VSMC migration is also linked to the cell cycle and proliferation[35]. VSMC proliferation plays a critical role in neointimal hyperplasia through cellular expansion and extracellular matrix deposition[35]. Under normal conditions, quiescent VSMCs are maintained in a non-proliferative phase, but after vessel injury, restriction points in the cell cycle ensure orderly progression through the cell cycle[35].

VSMC remodelling begins with an extracellular stimulus that activates receptors located on the cell surface. These, in turn, transduce the external signal to several pathways, leading to a series of coordinated remodelling events that trigger cell migration, proliferation or both. Signalling pathways that are activated when exposed to mitogens, growth factors or peptides often trigger pathways that stimulate both proliferation and migration[37]. The normally quiescent VSMCs within the medial layer of the vessel wall are activated to migrate and proliferate in response to increased stimulatory growth factors and cytokines (PDGF, IL-1, IL-6, and TNF-α) and reduced endothelium-derived inhibitory factors (NO, heparin sulfate proteoglycan)[35]. Both extracellular signal-regulated kinase (ERK)1/2 and protein kinase B (Akt) are important downstream signalling molecules for inducing VSMC proliferation and migration[39].

2.3. Adipose tissue and adipokines

Adipose tissue is a highly dynamic endocrine organ that regulates insulin sensitivity, energy metabolism and vascular homeostasis and secretes a number of different molecules that can communicate with essentially every organ in the body[6]. Obese

adipose tissue is characterized by adipocyte hypertrophy and infiltration of inflammatory macrophages and lymphocytes, leading to the augmented production of pro-inflammatory adipokines and vasoconstrictors that induce endothelial dysfunction and vascular inflammation through their paracrine and endocrine actions[6]. It has a central role in the development of obesity-associated metabolic complications as well as for low-grade systemic inflammation and increased risk of CVD[29].

The main functions of adipose tissue include the storage of triglycerides under conditions of excess calories and their release during periods of fasting, thermoregulation, and mechanical organ protection. The adipose tissue comprises ~50% adipocytes and ~50% other cells, including stem/precursor cells, preadipocytes, vascular, neural, immune cells and leucocytes[29]. Both the adipocytes and preadipocytes and, in particular, the macrophages/immune cells within the adipose tissue contribute to the release of metabolites, lipids, and bioactive peptides, so-called "adipokines", including IL-6, IL-8, IL-1b and monocyte chemoattractant protein (MCP)-1 (or CCL2)[29, 40]. Several adipokines regulate important biological processes in target organs, including the brain, liver, skeletal muscle, vasculature, heart, immune system and pancreatic β-cells[40]. Adipokines may exert specific effects on a variety of biological processes, including immune response (e.g., adipsin, acylating simulation protein (ASP), and serumamyloid A3 (SAA3)), inflammation (e.g., IL-1β, -6, -8, and -10, TNF-α, C-reactive protein (CRP), MCP-1, resistin, progranulin, and chemerin), glucose metabolism (e.g., leptin, adiponectin, dipeptidyl peptidase-4 (DPP-4), fibroblast growth factor 21 (FGF21), resistin, vaspin, and angiopoietin-like protein 8 (Angptl8)), insulin sensitivity (e.g., leptin, adiponectin, chemerin, retinol binding protein 4 (RBP4), and omentin), insulin secretion (e.g., apelin and nesfatin-1), blood pressure (e.g., apelin

and angiotensinogen), myocardial contractility (e.g., fatty acid binding protein-4 (FABP4)), cell adhesion (e.g., plasminogen activator inhibitor-1 (PAI-1)), vascular growth and function (e.g., VEGF), adipogenesis and bone morphogenesis (e.g., BMP-4 and -7), growth (e.g., insulin-like growth factor-1 (IGF-1), transforming growth factor β (TGFβ), and fibronectin), lipid metabolism (e.g., solubleCD36 and apelin), lipid accumulation in the liver (e.g., fetuin-A), regulation of appetite and satiety (e.g., leptin and vaspin), and other biological processes (Table 1)[40, 41].

Adipokine	Main actions
Adiponectin	Improves insulin sensitivity; antidiabetic, antiatherogenic, and anti-inflammatory
Adipsin	Activates the alternative complement pathway
Angptl8	Promotes pancreatic β cell proliferation; improves glucose tolerance
Apelin	Inhibits insulin secretion
BMP-4	Regulates adipogenic precursor cell commitment and differentiation
BMP-7	Stimulates brown adipogenesis; reduces food intake; increases energy expenditure
Cathepsins S, L, K	Regulate glucose metabolism and adipose tissue mass
Chemerin	Chemoattractant protein; regulates adipogenesis
Clusterin	Promotes tumor progression and angiogenesis
DPP-4	Degrades GIP and GLP-1; inhibitors in clinical use for T2DM
FABP4	Associated with increased T2DM risk and impaired myocardial contractility
Fetuin-A	Reflects liver fat content; associated with lipid-induced inflammation and IR; promotes cancer progression
FGF21	Stimulates glucose uptake into adipocytes; increases thermogenesis, energy expenditure, and fat utilization; improves glucose and lipid metabolism

(Continued) Adipokine	Main actions
Gremlin-1	Inhibits BMP-4 and BMP-7
IL-1β	Pro-inflammatory
IL-6	Pro-inflammatory
Leptin	Satiety signal; regulates appetite, food intake, locomotor activity, energy expenditure, fertility, and other processes
Lipocalin 2	Related to IR and inflammation
MCP-1	Chemoattractant protein; adipose tissue inflammation
Nesfatin-1	Direct glucose-dependent insulinotropic effect on β cells
Omentin	Anti-inflammatory; insulin sensitizing
Progranulin	Chemoattractant protein; neurodegenerative diseases; adipose tissue inflammation
RBP4	Related to IR, visceral fat distribution, and dyslipidemia
Resistin	Related to obesity, IR, and inflammation
TGFβ	Regulates cell proliferation, differentiation, and apoptosis
Tissue inhibitor of matrix metalloproteinase-1	Decreases adipogenesis; impairs glucose tolerance
TNF-α	Pro-inflammatory
Vaspin	Serine protease inhibitor; decreases food intake; improves hyperglycemia
VEGF	Stimulates angiogenesis in adipose tissue
Visfatin/PBEF/Nampt	Nampt-mediated systemic NAD biosynthesis is critical for β cell function
Wnt1 inducible signalling pathway protein 1	Regulates adipogenesis and adipose tissue inflammation

Table 1. Adipokines and their main actions[40].

The expansion of adipose tissue is a beneficial, adaptive response to overnutrition that can prevent ectopic lipid deposition and lipotoxicity in other cell types[42], and it is principally a consequence of adipose cell enlargement and/or recruitment of new cells. An increase in adipocyte cell size (hypertrophy) is often preceded by an

increase in cell number (hyperplasia), but with increasing obesity, the heterogeneity of the adipocytes is diminished and the population of hypertrophic cells increases (Fig. 3)[43]. Typical symptoms of adipose tissue dysfunction are visceral (ectopic) fat accumulation, changes in the cellular and intracellular matrix composition of adipose tissue, increased number of immune cells within adipose tissue, adipocyte hypertrophy, increased autophagy and apoptosis, adipose tissue extracellular matrix changes (adipose tissue fibrosis), alterations in adipose tissue mRNA and protein expression patterns[44]. Furthermore, under conditions of adipose tissue dysfunction, which is frequently found to accompany obesity, secretion of adipokines is dysregulated. Altered adipokine secretion may contribute to impaired regulation of appetite and satiety, fat distribution, insulin secretion and sensitivity, energy expenditure, endothelial function, inflammation, blood pressure, and hemostasis[44].

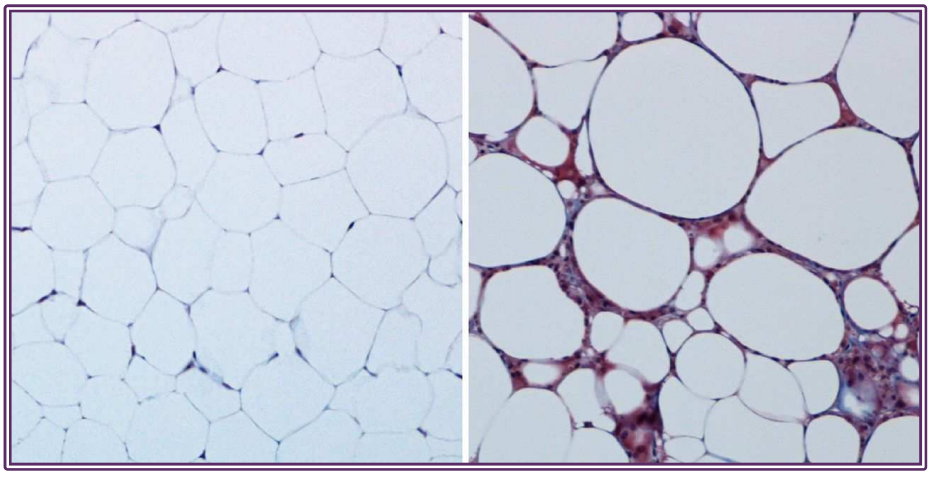

Figure 3. Benign (left) and unhealthy (right) adipose tissue[42].

The term "perivascular adipose tissue" (PVAT) refers to adipose tissue around vessels, irrespective of location. Known locations of PVAT include the coronaries (epicardial adipose tissue), aorta (periaortic adipose tissue), and the microvascular beds of the mesentery, muscle, kidney, and adipose tissue[45]. PVAT is a conglomerate of various cell types, including adipocytes, preadipocytes, and mesenchymal stem cells embedded in a matrix that is invested with microvessels[46]. Similar to visceral and subcutaneous adipose tissues, PVAT expands during obesity with hypertrophy of both brown and white periaortic adipocytes noted in rodent models of high-fat feeding[46].

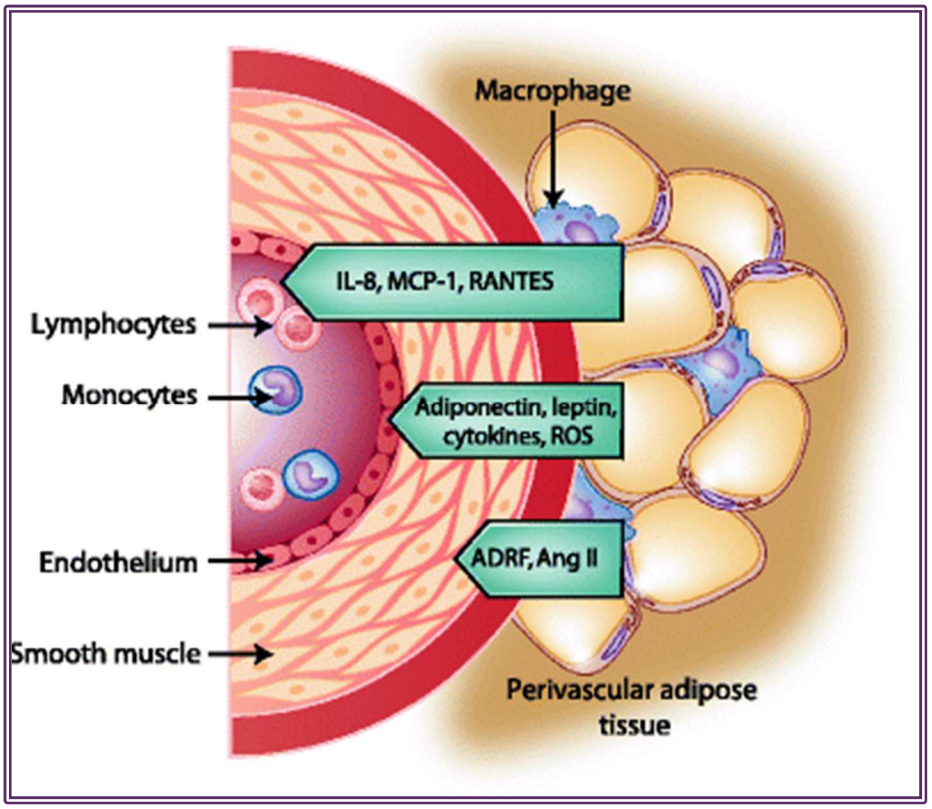

Figure 4. Interaction of PVAT with vascular endothelium, smooth muscle, and immune cells and the mediators involved[46].

The endothelium is an important modifier of vascular tone, and recent evidence suggests that PVAT alters the balance between endothelium-dependent vasodilator and vasoconstrictor substances, such as NO and endothelin-1. PVAT could modify vascular reactivity by secreting adipokines (Fig. 4), some of which are known to have an effect on vasoreactivity[45]. Furthermore, PVAT is anatomically colocalized with atherosclerotic lesions in humans, correlating with plaque burden and vascular calcification[46].

2.3.1. Adipokines in endothelial dysfunction

Adipose tissue, which is a highly vascularized organ, especially PVAT, exerts profound effects on vascular tone, in particular, endothelium-dependent vasodilatation, inflammation and remodelling by secreting a large number of bioactive substances, including adipocyte-derived relaxing factor (ADRF), vasoconstrictors and various cytokines and chemokines. The aberrant production of these factors is an important contributor to obesity-associated endothelial dysfunction and vascular inflammation[6].

Adiponectin is one of the most abundant adipokines secreted from adipocytes, accounting for approximately 0.01% of the total protein content of human plasma (3–30 μg/ml)[6, 47]. In endothelial cells, adiponectin enhances eNOS activity and NO production via AMP-activated protein kinase (AMPK)-mediated phosphorylation of eNOS at Ser^{1177} [6]. It reduces TNF-α-stimulated expression of endothelial adhesion molecules and monocyte attachment and attenuates the production of ROS induced by high glucose, oxidized LDL and palmitate[41]. The dysregulation of adiponectin production may be an important factor in endothelial dysfunction[47].

Leptin was the first adipokine to be characterized; it is a 16-kDa peptide hormone encoded by the *ob* gene and mainly produced by white adipose tissue, regulated by energy level, food intake, several hormones and various inflammatory mediators[47]. Leptin induces relaxation of the aorta and mesenteric arteries by inducing eNOS activity and NO release and also by enhancing the production of endothelium-derived hyperpolarizing factor[6, 48]. It also induces oxidative stress by increasing the formation of ROS, a key mediator of endothelial dysfunction reducing the bioavailability of NO in endothelial cells that leads to endothelial dysfunction[47].

Omentin promotes activation of eNOS and, therefore, promotes endothelium-dependent vasorelaxation and angiogenesis and prevents pro-inflammatory signalling in endothelial cells. Decreased omentin levels have been linked to endothelial dysfunction in humans[40].

Resistin (or adipose tissue-specific secretory factor (ADSF) or C/EBP-epsilon-regulated myeloid-specific secreted cysteine-rich protein (XCP19)) is a 12.5-kDa protein encoded by the *RETN* gene. Some studies have suggested that it may contribute to vascular disease through endothelial dysfunction mediated by reduced eNOS expression and NO production via oxidative stress and activation of p38 and c-Jun NH_2-terminal (JNK) mitogen-activated protein kinase (MAPK) (JNK MAPK). In addition, resistin increases the levels of vascular cell adhesion molecule (VCAM)-1 and MCP-1, whereas it downregulates TNF receptor-associated factor (TRAF-3), which is a powerful inhibitor of CD40 ligand-mediated endothelial cell activation[48].

Visfatin is a 52-kDa adipokine whose circulating levels were reported to be higher in obesity. It appears to mediate vascular endothelial inflammation by inducing the expression of adhesion molecules (VCAM-1 and intercellular adhesion molecule (ICAM)-1) via oxidative stress-dependent nuclear factor kappa B (NF-κB) activation

and directly induces endothelium-dependent vasorelaxation in rat aorta and mesenteric arteries mediated by NO production (dependent on the activation of phosphatidylinositol 3-kinase (PI3K)/Akt/eNOS pathways)[47].

FABP4 is one of the most abundant proteins in mature adipocytes[49]. It is secreted in the blood and is believed to play a central role in obesity-related CVD, possibly by potentiating lipid-induced inflammation. Plasma FABP4 levels are positively correlated with endothelial dysfunction, and it may contribute to endothelial dysfunction by potentiating lipid-induced impairment of eNOS activation[25]. Chronic treatment of *Apoe*$^{-/-}$ mice with an FABP4 inhibitor improved endothelium-dependent relaxations in the aorta studied *in vitro* without affecting endothelium-independent relaxations[50].

2.3.2. Adipokines in vascular smooth muscle cell proliferation and migration

VSMCs represent one of the major cell types of the artery wall that preserves vessel wall homeostasis. The migration of VSMCs from the media to intima and their concomitant proliferation occurring in the synthetic state are critical causes of arterial wall thickening. Some adipokines have been shown to affect the vasculature by influencing the proliferation and function of VSMCs[51]. It was demonstrated that lipid mediators and adipokines synergistically disturb VSMC function, inducing augmented proliferation and inflammatory signalling[51].

Adiponectin strongly suppressed *in vitro* VSMC proliferation and migration through direct binding with PDGF, suggesting that adiponectin acts as a modulator for vascular remodelling[52].

Leptin is believed to cause atherosclerosis by promoting the proliferation and migration of VSMCs, which in turn induces neointimal growth[53]. Leptin induces the proliferation of VSMCs by a mechanism involving the stimulation of PI3K activity, activation of MAPK, and progression to S and G2/M phases. It promotes the migration of VSMCs by activating the Rho/ROCK pathway, which promotes reorganization of the actin cytoskeleton. Leptin further leads to neointimal growth by stimulating platelet aggregation, activating monocytes, and regulating the immune response[53]. Exogenous leptin, administered at levels comparable to those found in obese humans, promoted neointimal hyperplasia and significantly increased the dose of rapamycin required for effective inhibition of neointimal formation[35]. Leptin-induced p38 MAPK activation is associated with the onset of hypertrophy and programmed cell death in rat VSMCs[53]. It activates the mTOR signalling pathway in primary murine VSMCs, stimulating proliferation *in vitro*[54].

Resistin plays an important role in VSMC migration, promoting VSMC adhesion and spreading[55]. Furthermore, it induces VSMC proliferation *in vitro* through both ERK1/2 and Akt signalling pathways[56].

2.3.3. Circulating adipokines and peripheral tissues

Adipokines function as classic circulating hormones to communicate with other organs, including brain, liver, muscle, the immune system, and adipose tissue itself (Fig. 4)[57]. The dysregulation of adipokines has been implicated in obesity, T2DM and CVD. Recently, inflammatory responses in adipose tissue have been shown as a major mechanism to induce peripheral tissue IR[57]. These circulating adipokines usually interact with peripheral tissues through plasma membrane receptors (Fig. 5 and Table 2).

Adipokine	Receptor
Adiponectine	AdipoR1, AdipoR2, T-cadherin
Leptin	LRa-f
Resistin	ΔDCN
TNF-α	TNFR-1,TNFR-2
Il-6	IL-6R, gp130
RBP4	STRA6, RBPR2
Chemerin	CMKLR1
FABP4	Unknown

Table 2. Adipokines and their receptors.

Adiponectin is a pleiotropic adipokine that exerts anti-inflammatory, antidiabetic, and antiatherogenic effects through its receptors (AdipoRs) AdipoR1 and AdipoR2[58]. AdipoR1 is found in many tissues, being particularly abundant in skeletal muscle, whereas AdipoR2 is most commonly found in the liver[59]. AdipoR1 and AdipoR2 modulate FA metabolism in the liver[59]. Cardiac myocytes and heart tissue express adiponectin receptors, which are decreased in hyperinsulinemia related to obesity[59]. A decrease in AdipoR1 also decreases AMPK-dependent angiogenic response, and downregulation of the adiponectin receptor pathway may be causally related to a decrease in cardiovascular function. Adiponectin receptors are also expressed in gastric, breast, prostate and endometrial cancer cells[59]. RPPAR and AMPK are downstream mediators of AdipoR1 and AdipoR2 that increase

inflammatory responses by trans-repression of NF-κB target genes, including COX2, which may partly account for the anti-inflammatory effects of adiponectin[59]. In addition, macrophage polarization contributes to differential AdipoR expression and contrasting inflammatory responses to adiponectin[58]. T-cadherin is another adiponectin receptor that is expressed in endothelial and smooth muscle cells[60], and its role in adiponectin signalling appeared to be minor because, in contrast to control mice, administration of adiponectin to *AdipoR1/R2* double knockout mice did not improve plasma glucose levels[57]. Exogenous administration of adiponectin or overexpression in transgenic mice results in improved insulin sensitivity, whereas adiponectin-deficient mice develop high fat diet-induced inflammation and IR[57].

Leptin is a pleiotropic cytokine that acts as a circulating hormone, regulating appetite and the expenditure of energy via its action on specific receptors expressed in the hypothalamus with activities on many peripheral cell types[61, 62]. Additional pleiotropic effects of leptin include modulation of immune responses, angiogenesis, neovascularization and bone formation[62]. Inappropriate leptin signalling can promote autoimmunity, certain CVDs, elevated blood pressure and cancer[61]. The levels of leptin in plasma are proportional to fat mass and increase as body weight rises. In humans, leptin circulates as a free hormone or bound to a soluble leptin receptor (LR). The full-length LR is divided into three regions: an extracellular part, a single-pass helix trans-membrane domain and an intracellular part[63]. Six LR isoforms produced by alternative splicing or proteolytic ectodomain shedding have been identified (LRa-f). LRb is highly expressed in specific nucleus of the hypothalamus, a region of the brain that is known to be involved in regulating body weight. However, LRb expression could be shown in a broad range of other cell types, thereby explaining the pleiotropic effects of leptin[61]. Leptin-deficient (*ob/ob*) and LR-deficient (*db/db*) mice display marked hyperphagia,

obesity, and IR. Importantly, exogenous administration of leptin to *ob/ob* mice reduces obesity and restores insulin sensitivity. However, leptin levels in circulation are increased in obese rodents and humans, suggesting that obese subjects display leptin resistance[57]. Leptin administration has been proven effective in animals and patients with lipodystrophy, a disease characterized by almost an absence of fat mass and therefore low secretion of leptin, resulting in excessive caloric intake, which is stored as fat in liver and muscle, leading to T2DM and high blood lipid levels or for the treatment of anorexia nervosa[64]. It also significantly reduces triglycerides, transaminases, hepatomegaly, and liver fat content. These reductions are associated with significant reductions in steatosis and the hepatocellular ballooning injury seen in non-alcoholic steatohepatitis[65], and it was demonstrated that its administration increased AMPK activities in the liver as well as in skeletal muscle with significant reduction in triglyceride content[66].

Resistin is a potential link between obesity and IR or T2DM. In rodents, resistin is primarily expressed in and secreted from mature adipocytes with some expression in pancreatic islets and portions of the pituitary and hypothalamus. Its secretion can be upregulated by several factors, including insulin and glucose[67]. An isoform of decorin (ΔDCN) was identified as a resistin receptor on the surface of adipose progenitor cells[68]. Expression of this isoform in 3T3-L1 cells promoted proliferation and migration but suppressed lipid accumulation upon adipogenesis induction, which was resistin dependent. ΔDCN serves as a functional receptor of resistin in adipocyte progenitors and may regulate white adipose tissue expansion[68]. Resistin is involved in the activation of SOCS3, resulting in the suppression of insulin-mediated signalling in adipocytes. Thus, resistin deficient *ob/ob* mice show improved glucose tolerance and insulin sensitivity[57]. In contrast, the function of resistin in humans is not clear, as resistin levels in blood circulation are not

correlated with obesity or IR[57]. It is known that resistin is also an immune-derived systemic and locally acting pro-inflammatory cytokine[69].

TNF-α and IL-6 are the most widely studied cytokines produced by adipose tissue[57]. TNF-α is an inflammatory cytokine mainly produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis[70]. In humans, adipose tissue TNF-α expression correlated with body mass index (BMI), percentage of body fat, and hyperinsulinemia, whereas weight loss decreased TNF-α levels. Fasting TNF-α plasma levels were associated with IR in the Framingham Offspring Study, and its neutralization in obese rats improved IR[57]. It is demonstrated that TNF-α blocks the action of insulin through its ability to inhibit insulin receptor tyrosine kinase activity[71]. The protein is also important for resistance to infection and cancers[70]. TNF-α exerts many of its effects by binding to two cell membrane receptors, termed TNFR-1 and TNFR-2[70].

IL-6 is produced by various types of cells, influences various cell types, and has multiple biological activities through its unique receptor system. IL-6 exerts its biological activities through two molecules: IL-6R (IL-6 receptor) and gp130[72]. Adipose tissue contributes 10–35% of circulating IL-6 levels in humans, and hypertrophic enlargement of adipocytes is accompanied by increased production of IL-6 by adipose tissue whose expression positively correlates with IR both *in vivo* and *in vitro*. IL-6 plays an important role in the development of IR in obesity[73]. IL-6 was reported to reduce insulin-dependent hepatic glycogen synthesis and glucose uptake in adipocytes, whereas insulin-dependent glycogen synthesis and glucose uptake was enhanced in myotubes[57].

RBP4 is expressed in the liver, adipocytes, and macrophages[74]. In adipocytes, its expression is inversely correlated with GLUT4[57]. RBP4 circulating levels have been

shown to correlate with obesity and IR in rodents[75], and it is known that administration of recombinant RBP4 to normal mice induces IR[58]. In humans, RBP4 levels have been shown to be elevated in several groups of insulin-resistant subjects, but not all studies have shown such differences[75]. Circulating RBP4 and its membrane receptor STRA6 coordinate cellular retinol uptake[74] but not in liver as STRA6 is not expressed in that tissue[76]; RBP4 receptor-2 (RBPR2) is a retinol transporter expressed primarily in liver and intestine and induced in the adipose tissue of obese mice[76].

Chemerin mediates inflammatory responses and induces the infiltration of macrophages, immature dendritic cells, and natural killer (NK) cells in inflammatory diseases, such as ulcerative colitis and skin lupus, and regulates adipogenesis and adipocytes[57]. Chemerin level is positively correlated with BMI, fasting glucose, triacylglycerols, and inflammatory cytokines in obese subjects, and administration of chemerin exacerbates glucose intolerance in obese mice[57]. Chemerin is the natural ligand of the G protein-coupled receptor ChemR23 (CMKLR1), but it has more receptors, such as GPR1 and CCRL2. These three receptors are expressed by adipocytes[77]. Chemerin/CMKLR1 regulates adipogenesis and osteoblastogenesis of bone marrow-derived precursor cells[78]. Furthermore, CMKLR1 is upregulated in the brains of Alzheimer's disease patients[79].

FABP4 is one of the most abundant proteins in adipocytes, and it plays a crucial role in FA uptake[80]. Recently, it has been shown to play a role in FA uptake into muscle and ovarian cancer cells. Secreted FABP4 potentiates hepatic glucose production and insulin secretion, reinforcing the role of this protein in metabolic homeostasis[80]. Epidemiological studies demonstrate a close association between serum levels of FABP4 and a cluster of obesity-related cardiometabolic risk factors,

endothelial dysfunction, and macrovascular complications of T2DM[81]. In addition to its role in lipid metabolism and insulin sensitivity, both clinical investigations and animal studies suggest that FABP4 is a central player in mediating obesity-related vascular disease, primarily by inducing IR and potentiating lipid-induced inflammation[81]. However, the mechanism of action of this adipokine remains unclear, and its cell interaction is unknown. To date, neither the FABP4 ligand nor the receptor has been identified at the cellular level.

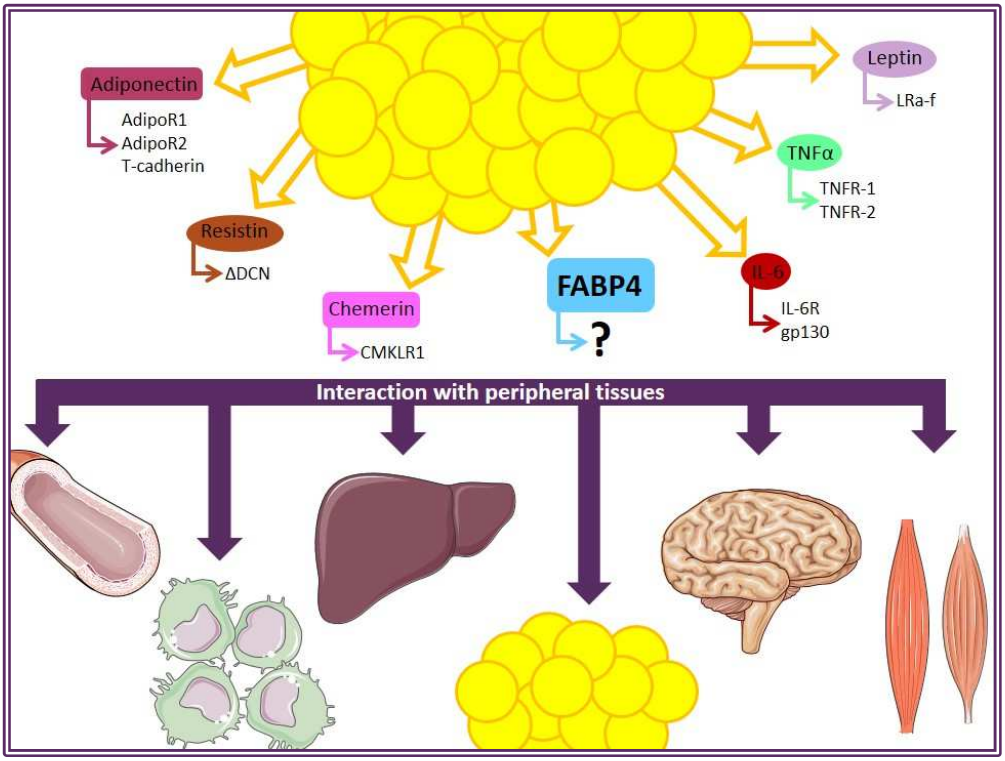

Figure 5. Interaction of adipokines with peripheral tissues.

2.4. FABPs

Fatty acid-binding proteins (FABPs) are a family of highly expressed intracellular lipid-binding proteins (iLBPs) formed by small (14–15 kDa) cytoplasmic proteins that bind reversibly with high affinity to hydrophobic ligands, such as saturated and unsaturated long chain fatty acids (LCFA), eicosanoids and other lipids. FABPs can be found across all species, demonstrating strong evolutionary conservation[82, 83]. Hydrophobic ligands, such as fatty acids (FAs) and their acyl-CoA derivatives (FA-CoA), serve many biological functions within the cell[84-86]. They are important sources of energy stored in triacylglycerol and are produced in muscles and the liver. They are also used for the formation of complex lipids, such as phospholipids and cholesterol. Finally, FA residues are hormones and signalling compounds, as they modify specific proteins. These essential nutrients are obtained from one's diet, released from adipocytes, or synthesized from glucose in the liver[87]. The insoluble properties of FAs means they require chaperones to bind and transfer them throughout various cellular compartments, including the peroxisomes, mitochondria, endoplasmic reticulum (ER), lipid droplets and nucleus[85].

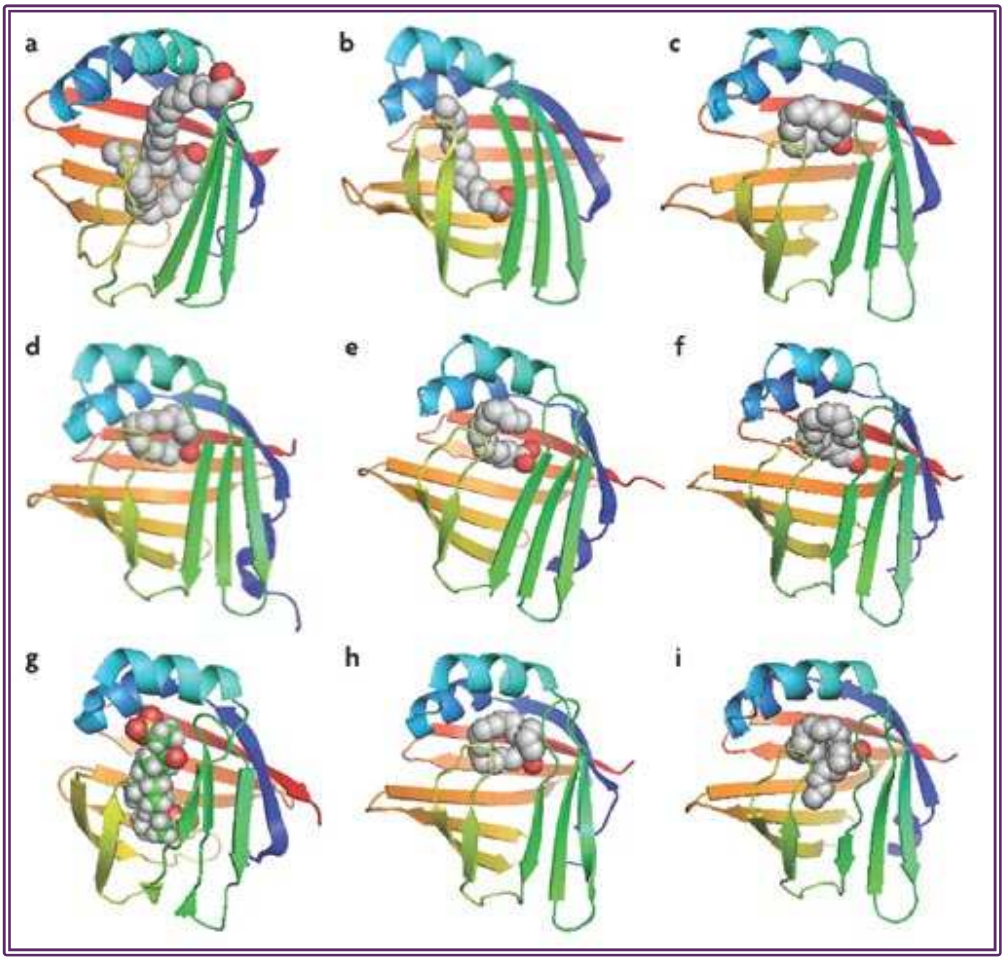

Figure 6. Crystal structure of ligand-bound FABPs[86]. (a) FABP1 (L-FABP). (b) FABP2 (I-FABP). (c) FABP3 (H-FABP). (d) FABP4 (A-FABP). (e) FABP5 (E-FABP). (f) FABP8 **(M-FABP). (g) FABP6 (Il-FABP). (h)** FABP7 **(B-FABP). (i) A long-chain docosahexaenoic acid in a helical conformation accommodate in a FABP binding pocket[86].**

FABPs are abundant intracellular proteins expressed in almost all tissues; nine separate genes have been identified in mammals. FABPs are named after the tissue in which they were discovered or are prominently expressed. This nomenclature can be misleading because several FABPs are expressed in more than one tissue, and a numerical nomenclature for the various FABPs has been introduced[88, 89]. The

family contains liver (L-), intestinal (I-), heart (H-), adipocyte (A-), epidermal (E-), ileal (Il-), brain (B-), myelin (M-) and testis (T-) FABPs[86]. There is a new gene, *FABP12*, of the family, providing further evidence of the evolutionary divergence of the FABP protein family from a single ancestral gene, as it is hypothesized to have formed from a tandem gene duplication[85]. FABPs have an extremely wide range of sequence diversity: from 15 to 70% sequence identity between different members[87]. Despite modest amino acid sequence homologies, the FABPs exhibit very similar tertiary structures. Their 10 anti-parallel β-strands are organized into 2 nearly orthogonal β-sheets that form a slightly elliptical β-barrel with two 8-10 residue helixes linking the first two β-strands[89-91]. The binding pocket is located inside the β-barrel, the opening of which is framed on one side by the N-terminal helix-loop-helix 'cap' domain and FAs bound to the interior cavity. There is a conserved three-element fingerprint that provides a signature for all FABPs (Fig. 6 and Table 3)[86].

Numerous functions have been proposed for FABPs. Their presence in the cell is essential for the binding of hydrophobic molecules, reducing the detergent-like properties of high FA concentrations and making them more soluble[85]. As lipid chaperones, FABPs may actively facilitate the transport of lipids to specific compartments in the cell, such as to the lipid droplet for storage; to the ER for signalling, trafficking and membrane synthesis; to the mitochondria or peroxisome for oxidation; to cytosolic or other enzymes to regulate their activity; to the nucleus for lipid-mediated transcriptional regulation (it has been demonstrated that FABPs can target FAs to transcription factors, such as the peroxisome proliferator-activated receptor (PPAR) family in the lumen of the nucleus) or even outside the cell to signal in an autocrine or paracrine manner[85, 86].

Gene	Common name	Aliases for proteins	Previous symbols	Localisation
FABP1	Liver FABP	L-FABP, hepatic FABP, Z protein, homo-binding protein		Liver, intestine, pancreas, kidney, lung, stomach
FABP2	Intestinal FABP	I-FABP, gut-FABP (gFABP)		Intestine, liver
FABP3	Heart FABP	H-FABP, O-FABP, mammary-derived growth inhibitor (MDGI)	FABP11	Cardiac and skeletal muscle, brain, kidney, lung, stomach, testis, adrenal gland, mammary gland, placenta, ovary, brown adipose tissue
FABP4	Adipocyte FABP	A-FABP, aP2		Adipocytes, macrophages, dendritic cells, skeletal muscle fibres
FABP5	Epidermal FABP	E-FABP, keratinocyte-type FABP (KFABP), psoriasis-associated-FABP (PA-FABP)		Skin, tongue, adipocyte, macrophage, dendritic cells, mammary gland, brain, stomach, intestine, kidney, liver, lung, heart, skeletal muscle, testis, retina, lens, spleen, placenta
FABP6	Ileal FABP	IL-FABP, ileal lipid-binding protein (ILBP), intestinal bile acid-binding protein (I-BABP), gastrophin		Ileium, ovary, adrenal gland, stomach
FABP7	Brain FABP	B-FABP, brain lipid-binding protein (BLBP), MRG		Brain, central nervous system, glial cell, retina, mammary gland
FABP8	Myelin FABP	M-FABP, peripheral myelin protein (PMP2)		Pheripheral nervous system, Schwann cells
FABP9	Testis FABP	T-FABP, testis lipid-binding protein (TLBP), PERF, PERF 15		Testis, salivary gland, mammary gland
FABP12	-	-		Retinoblastoma cell,[a] retina (ganglion and inner nuclear layer cells),[b] testicular germ cells,[b] cerebral cortex,[b] kidney,[b] epididymis[b]

Table 3. Human *FABP* genes, as listed in the Human Gene Nomenclature Committee (HGNC) and Online Mendelian Inheritance in Man (OMIM) databases. [a]Expression found in humans, versus [b]rodents[85].

2.4.1. FABP1 (L-FABP)

FABP1 is abundantly expressed in the liver, accounting for up to 5% of the total cytosolic protein. The liver isoform of the FABP family of proteins is unique, attributed to its ability to bind multiple ligands at once[85], it is the only FA-binding member of the mammalian FABP family to transfer FAs to membranes by aqueous diffusion[89]. The high level of expression, binding properties and function in regulating a variety of cellular processes (inflammation, immunity, metabolism and energy homeostasis) demonstrate the importance of L-FABP[85].

2.4.2. FABP2 (I-FABP)

FABP2 is highly expressed throughout the intestine with the highest levels in the distal portion of the organ and mediates fat absorption through binding and intracellular trafficking of free LCFAs[85].

2.4.3. FABP3 (H-FABP)

FABP3 constitutes 4–8% of the cytosolic protein in the mammalian heart and is highly expressed in both cardiac and skeletal muscle and, to a lesser extent, in stomach, brain, lung and mammary gland[85]. It mediates the passage of FAs from the plasma membrane to sites of lipid synthesis and is a potent inducer of cardiac myocyte hypertrophy, stimulating an increase in cell surface area, protein synthesis and c-jun expression[92]. A high affinity receptor for H-FABP has been identified[92].

2.4.4. FABP4 (A-FABP)

FABP4 is significantly expressed in white and brown adipose tissue, monocytes and macrophages[85], and it is also found in endothelial cells[93]. In addition to the chaperone-like activity responsible for integrating lipid signals and organelle responses, FABP4 interacts with HSL, potentially modulating its catalytic activity and integrating signalling networks that control inflammatory responses and lipid hormone production in adipocytes. In macrophages, FABP4 also mediates inflammatory responses through the inhibitor of kappa kinase nuclear factor kB (IKK-NF-kB) and JNK-activator protein 1 (AP-1) pathways and attenuates cholesterol efflux through inhibition of the PPARγ–liver X receptor a (LXRa)–ATP binding cassette A1 (ABCA1) pathway[85].

2.4.5. FABP5 (E-FABP)

FABP5 is expressed in skin, liver, brain, lung, and cancerous tissue and appears to play tissue-specific roles in each[89]. It is difficult to decipher the exact functionality of the protein (in addition to its generic role in FA binding and trafficking) because all of these tissues also express additional FABPs. Gene ablation of *Fabp4* in adipocytes results in a significant upregulation of FABP5 expression, and ablation of both genes in mice suggests importance in systemic glucose and lipid homeostasis[85].

2.4.6. FABP6 (Il-FABP)

FABP6 binds biliary acids (BAs) and FAs (although with less affinity) and interacts with the ileal BA transporter protein at the cytoplasmic face of the ileocyte to aid

in uptake and trafficking of BAs. The ileum plays a critical role in the enterohepatic circulation of BAs, further demonstrating its importance for FABP6 and for specificity for binding and trafficking BAs[85].

2.4.7. FABP7 (B-FABP)

Tissue expression of FABP7 is both spatially and temporally correlated with neuronal differentiation in many regions of the central nervous system, including the postnatal cerebellum, embryonic spinal cord and cerebral cortex. It exhibits higher affinity towards FAs with longer chain lengths with n-3 polyunsaturated FAs (docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and a-einolenic acid) being the preferred ligands[85].

2.4.8. FABP8 (M-FABP)

FABP8 is one of the major proteins comprising peripheral nervous system myelin[85] and is an extrinsic membrane protein; thus, its membrane interaction properties, seen to some extent with all collisional transfer FABPs, are likely to be substantial and may dictate its function in peripheral nerve myelin[89].

2.4.9. FABP9 (T-FABP)

FABP9 is a poorly understood member of the FABP family. It is expressed during spermatogenesis and in mammalian testis. It is known that the FA composition of sperm is related to fertility, so the proposed function of T-FABP has been

hypothesized to protect sperm FAs from oxidation, thus maintaining their ability to fertilize oocytes[85].

2.4.10. FABP12

The *FABP12* gene is the newest member of the FABP family and, to date, little information is available on the functional properties of the protein. FABP12 is phylogenetically restricted; it has been identified in human, rat and mouse, but no counterpart has been identified in chicken or zebrafish genomes[85].

2.5. Fatty acid binding protein 4 (FABP4)

2.5.1. Regulation

Fatty acid-binding protein 4 (FABP4; adipocyte-FABP; aP2) is a member of the family of intracellular FABPs[82]. It is the major FABP in adipose tissue[26], and it is also highly expressed in macrophages, having a role in lipid metabolism in both cell types[28]. It is also expressed in monocytes[85] and endothelial cells[93].

FABP4 is located on Chr 8q21 and is regulated by a number of cis-acting regulatory elements and trans-acting nuclear factors in the 50-bp flanking region of the genes that activates or represses transcription[85, 94]. Two fat-specific elements (FSE) are found within the FABP4 gene, including four copies (three direct and one inverted) of the 13-bp FSE1 (5'-GGCTCTGGTCATG-3') and 15-bp FSE2 (5'-ACTCAGAGGAAAAG-3')[85]. The canonical TATA box is located 31 bp upstream of the transcription start site. Additionally, the gene has overlapping positive (AP-1) and negative (cAMP) regulatory elements with a binding sequence for the trans-activating C/EBP upstream of these regulatory elements[94]. Further upstream of the 50-bp flanking

region of FABP4 are five cis-acting adipocyte regulatory elements (ARE1, ARE2, ARE4, ARE6 and ARE7)[85]. The ARE1 site targets a member of the nuclear factor (NF) 1 family and, when mutated, reduces the activity of the enhancer in adipocytes by 76%. Both ARE2 and ARE4 are recognized by adipocyte regulatory factor 2 (ARF2), stimulating promoter activity in several cell types, whereas ARE6 and ARE7 stimulate promoter activity only in adipocytes and are bound by adipocyte regulatory factor 6 (ARF6)[94]. FABP4 also contains several functional putative PPAR response elements (PPREs) approximately 5.5 kb upstream of the transcription start site, allowing for transcriptional regulation of FABP4 by FA, PPARγ, insulin and agonists of PPARγ, such as thiazolidinediones[83, 85, 95]. The nuclear import of FAs has been shown to involve FABP4 to activate PPARγ in adipocytes[96]. Nuclear translocation of FABP4 appears to depend on the stabilization of an "activated" state of FABP4 by a subset of small molecule ligands[97].

The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is a key component for cellular redox homeostasis in the attenuation of oxidative stress-associated pathological processes. In the cardiovascular system, patients with insufficient Nrf2 levels in multiple tissues are likely susceptible to several adverse components of disease development[98]. In mice, enhanced Nrf2 activity increased expression of *Fabp4* in the liver and decreased expression of *Fabp4* in white adipose tissue[99]. In adipocytes, this *Fabp4* decrease is mediated by NAD(P)H:quinine oxidoreductase 1 (NQO1), whose protein expression in mature adipocytes is regulated through Nrf2[100]. Furthermore, in macrophages, the human *FABP4* promoter contains an antioxidant response element (ARE) on the forward strand, and activated Nrf2 seems to be able to bind to this ARE, enhancing FABP4 expression[101]. Atorvastatin, a cholesterol-lowering drug, has been shown to reduce FABP4 expression in macrophages[102, 103]. Metformin, an antidiabetic drug, has also been reported to

inhibit forkhead box protein O1 (FOXO1)-mediated transcription of FABP4 and to reduce lipid accumulation in macrophages[103].

In macrophages, in response to pro-inflammatory stimuli, activated JNK increases FABP4 expression by inducing the phosphorylation of c-Jun, which in turn binds to a highly conserved AP-1 cis-element within the FABP4 gene promoter and enhances gene transcription. In addition, elevated FABP4 potentiates JNK phosphorylation and subsequent activation of the AP-1 complex, leading to elevated production of pro-inflammatory cytokines[104]. The reciprocal regulation between FABP4 and JNK is also supported by animal studies, demonstrating that genetic or pharmacological inhibition of FABP4 suppresses JNK activity in adipose tissue of obese mice as well as in atherosclerotic lesion areas of *Apoe*$^{-}$/$^{-}$ mice[81, 105, 106].

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) functions as a multifunctional lipid- and protein-specific phosphatase. Analysis of the gene expression profile in PTEN-null keratinocytes showed 5-fold elevated expression of FABP4, suggesting the importance of PTEN in the regulation of FABP4 expression at the level of transcription[107].

In endothelial cells, FABP4 is strongly regulated by VEGF and mTORC1, and several key angiogenic signalling pathways that are modulated by FABP4 in endothelial cells, including p38, eNOS, and SCF/c-kit, have been identified[108]. Furthermore, it is known that FABP4 induction by VEGFA is through a Delta-like ligand (DLL)4-NOTCH-dependent mechanism and may be modulated by Akt pathways via FOXO1[109]. VEGFA binding to VEGF receptor 2 (VEGFR2) on endothelial cells leads to increased DLL-4 on the cell surface, which is a ligand for the NOTCH receptor on adjacent cells. NOTCH intracellular domain (NICD) is released and translocates to the nucleus to regulate gene transcription[109].

2.5.2. Biological relevance

FABP4, as a member of the FABP family, is able to bind a variety of hydrophobic ligands[110-112108-110]; its endogenous ligands include oleic acid, retinoic acid, arachidonic acid, and 15-deoxy-D[12,14]-prostaglandin J2[81]. FABP4 is also involved in the conversion of FAs to eicosanoid intermediates and in the stabilization of leukotrienes[86]. As a lipid chaperone, FABP4 may actively facilitate the transport of lipids to specific compartments in the cell, such as to the lipid droplet for storage; to the ER for signalling, trafficking and membrane synthesis; to the mitochondria or peroxisome for oxidation; to cytosolic or other enzymes to regulate their activity; to the nucleus for lipid-mediated transcriptional regulation; or even outside the cell to signal in an autocrine or paracrine manner[86].

The first FABP mouse model created was the FABP4-deficent mouse (*Fabp4*$^{-}$/$^{-}$) model[113] that allowed for a direct examination of the presumed role of lipid-binding proteins in the mobilization and trafficking of intracellular FAs[114]. The biological relevance of FABP4 is underscored by the findings that *Fabp4*$^{-}$/$^{-}$ mice exhibit marked protection against IR, atherosclerosis, fatty liver disease, and asthma[111, 113, 115-118].

At baseline, the phenotype of *Fabp4*$^{-}$/$^{-}$ mice was unremarkable. The mice were healthy with no defects in adipose tissue, reproduction, or growth. It was surprising that the deletion of a protein that comprised up to 5% of the total cellular protein in adipocytes had no effect on fat development[49, 118]. The lack of a phenotypic change prompted a search for the expression of genes encoding other FABPs in adipocytes. In adipocytes, when *Fabp4* is silenced, *Fabp5* is upregulated 20–40-fold (mRNA) with the protein being increased 13-fold[119]. When the mice were stressed with diet-induced obesity, fasting plasma glucose, insulin and cholesterol levels were decreased relative to those of wild-type mice, even though the mice gained a

similar amount of weight. The serum triglycerides in obese wild-type mice were significantly elevated relative to those of obese *Fabp4*$^{-}$/$^{-}$ mice, whose serum triglycerides remained low, potentially because of an alteration in triglyceride synthesis and/or secretion. Glucose and insulin tolerance tests indicated the maintenance of insulin sensitivity in obese *Fabp4*$^{-}$/$^{-}$ mice in contrast to the IR that occurred in the wild-type mice. Thus, a single disruption in *FABP4* created a genetic model to unlink obesity and IR. Changes in lipid metabolism were manifested as a threefold increase in the total amount of free FAs in adipocytes[119]. Adipocytes from *Fabp4*$^{-}$/$^{-}$ mice exhibit diminished lipolysis and increased cellular FA levels[114, 117], and the *Fabp4*$^{-}$/$^{-}$ animals fed a high fat diet fail to develop the IR or T2DM normally associated with the ensuing obesity[113]. Expression of FABP4 is highly induced during adipocyte differentiation and transcriptionally controlled by PPARγ agonists, FAs, dexamethasone, and insulin[103]. A recent study in mice adipocytes showed a negative interaction between FABP4 and leptin and the opposing role that FABP4 and leptin play on mitochondrial FA oxidation[120]. FABP4 increased the expression of FA transport protein 1 (FATP1) and reduced FA transport protein (FAT) expression, indicating that FABP4 enhanced the FA transportation that is alleviated by leptin. It also activated key FA synthesis enzymes, such as FA synthase (FAS), and inhibited phosphorylation of acetyl-CoA carboxylase (ACC) while impairing the activity of FA oxidation key enzymes carnitine palmitoyl transferase-1 (CPT-1) and acyl-coenzyme A oxidase (AOX1)[120].

Expression of FABP4 is induced during differentiation from monocytes to macrophages and by treatment with lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate, PPARγ agonists, oxidized LDL, and advanced glycation end products[103] and impacts two key macrophage functions involved in atherogenesis: cholesterol trafficking and inflammatory activity[121, 122]. The proatherogenic role of FABP4 is

mediated by its direct actions on macrophages independent of lipid metabolism and insulin sensitivity[81]. Bone marrow transplantation of *Fabp4*$^{-}$/$^{-}$ in *Apoe*$^{-}$/$^{-}$ mice studies demonstrated that actions in macrophages showed alterations in inflammatory cytokine production and a reduced ability to accumulate cholesterol esters when exposed to modified lipoproteins and are predominant for the protective effect of FABP4 deficiency on atherosclerosis[123]. FABP4 also increases accumulation of cholesterol ester and foam cell formation via inhibition of the PPARγ-liver X receptor α (LXRα)-ABCA1 pathway and induces inflammatory responses through activation of the IKK-NF-κB and JNK-AP-1 pathways[104, 121].

In cultured endothelial cells, FABP4 mRNA and protein levels were significantly induced by VEGF-A and bFGF treatment. Thus, FABP4 is a target of the VEGF/VEGFR2 pathway and a positive regulator of endothelial cell proliferation[93] and angiogenesis-related functions, including survival, migration, and angiogenic sprouting *in vitro*[108].

Dendritic cells are potent antigen-presenting cells and play an essential role in the activation of naive T cells[124]. FABP4 in dendritic cells regulates the IKK-NF-κB pathway and T-cell priming, which might contribute to the development of atherosclerosis because both dendritic cells and T-cells are involved in the pathogenesis of atherosclerosis[103]. This provides an additional link between metabolic processes and the regulation of immune responses[124].

Keratinocyte-specific *Pten*$^{-}$/$^{-}$ mice revealed distinct phenotypes, including epidermal and sebaceous gland hyperplasia. The most induced gene in these mice was FABP4; thus, it is conceivable that the FABP4 pathway mediates the sebaceous gland hyperplasia in keratinocyte-specific *Pten*$^{-}$/$^{-}$ mice[125].

2.5.3. FABP4 interactions

The divergent sequences of the members confer subtle differences in their ligand binding properties and may also indicate different protein-protein interaction partners depending on the cellular context[28]. It was demonstrated that FABP4 physically associates with hormone-sensitive lipase (HSL) in a FA-dependent manner with high affinity and specificity (*Kd* = 0.5 nM) and that such an interaction represents a novel feed forward mechanism for regulating HSL activity[126]. It is also known that FABP4 interacts with Janus kinase 2 (JAK2) in a FA-dependent manner, establishing a new role for FABP4 as a FA sensor that affects cellular metabolism via protein-protein interactions[28].

In adipocytes, FABP4 interacts with PTEN, suggesting a role for this phosphatase in the regulation of lipid metabolism and adipocyte differentiation. The interaction between PTEN and FABP4 suggests a possible link between PTEN function and lipid metabolism and adipogenesis[107]. Additionally, it has already been shown that FABP4 interferes with the insulin receptor in adipocytes[127, 128]. It is known that FABP4 suffers a ligand-induced conformational change in FA-binding protein, leading to altered function. Upon binding FA, FABP4 undergoes a conformational change whereby Tyr19 becomes accessible for phosphorylation by the insulin receptor tyrosine kinase and to iodination by lactoperoxidase[127].

FABP4 forms a physical complex with the adipose triglyceride lipase (ATGL) co-activator CGI-58 both *in vitro* and in living cells[96]. The interaction of FABP4 and CGI-58 has important functional consequences. First, FABP4 promotes ATGL-mediated triglyceride hydrolysis. Consistent with these findings that FABP4 only binds to CGI-58 but not to ATGL, the activation of ATGL's hydrolytic activity by FABP4 depends on the presence of CGI-58. Apparently, CGI-58 acts as an adaptor protein, enabling FABP4 to bind the FAs produced by ATGL. This interaction represents the basis for

a novel mechanism for the modulation of lipolysis and PPAR-mediated gene expression[96].

2.5.4. FABP4 and oxidative stress

Oxidative stress is counterbalanced by complex antioxidant defence systems regulated by a series of multiple pathways to ensure that the response to oxidants is adequate[129]. Nrf2 is a regulator of cellular resistance to oxidants and stimulates the transcription of genes involved in many aspects of cytoprotection, most notably phase 2 detoxifying enzymes such as NQO1[130]. In mature adipocytes of mice, Nrf2 activity, through NQO1 protein[100], decreased triglycerides and free FAs content accompanied by decreased expression of FABP4[99]. The chronic accumulation of cellular stress in adipocytes contributes to the development of metabolic disorders such as T2DM and CVDs[131].

Various cellular perturbations implicated in the pathophysiology of human diseases, including CVD, T2DM and obesity, can alter ER function and lead to the abnormal accumulation of misfolded proteins. This situation configures the so-called ER stress, a form of intracellular stress that occurs whenever the protein-folding capacity of the ER is overwhelmed. In ER stress, accumulation of unfolded proteins is detected, and transcriptional and translational pathways that address unfolded and misfolded proteins are activated; this is known as the unfolded protein response (UPR)[129]. The UPR has been proposed to be a protective mechanism that limits ER protein loading by inhibiting protein translation, facilitating protein folding through increasing the expression of ER chaperones and removing misfolded proteins from the ER through degradation. However,

prolonged and unrestrained ER stress could lead to the activation of proapoptotic signalling pathways[129].

Atherosclerosis is associated with ER stress[129, 132]. Endothelial ER stress was found in areas of disturbed flow, and atherosclerosis develops almost exclusively in areas of disturbed flow (slow flow or low shear stress, often with eddy currents, characterized by slow, oscillating flow)[132]. Although data on FABP4 in human atherosclerosis are scarce, it has an important role in pathophysiological cascades of human atherosclerotic disease. Macrophages show ER stress when exposed to lipotoxic signals associated with atherosclerosis[105], and it is known that FABP4 expression is high in unstable carotid plaques and associates with the cardinal features of plaque vulnerability and with ER stress and increased apoptosis[17]. Furthermore, *Fabp4*$^{-}$/$^{-}$ mice show reduced macrophage apoptosis in atherosclerotic lesions *in vivo* through the alleviation of lipotoxic ER stress[105]. It has also been demonstrated that genetically reduced FABP4 expression within the atherosclerotic plaque may modulate ER stress signalling and attenuate apoptosis, lipid burden, and inflammation with the combined effect of promoting atherosclerotic plaque stability, suggesting an important role for FABP4 in pathophysiological cascades of human atherosclerotic disease[17].

However, recent studies in 3T3-L1 adipocytes have demonstrated that FABP4 has a role in alleviating oxidative and ER stress. The attenuation of these types of cellular stress via FABP4 might play a key role in the maintenance of adipocyte homeostasis because the excess level of cellular oxidative and end ER stress leads to adipocyte dysfunction to include impaired glucose/lipid metabolism and endocrine capacity[131]. In differentiated adipocytes, FABP4 is associated with lowering the level of intracellular ROS and acts as alternate antioxidant protein against H_2O_2[131]. These

results suggest a new role for FABP4 in cytoprotection against oxidative and ER stress.

The condition of oxidative stress arises when oxidant production exceeds antioxidant activity in cells and plasma. The overabundance of oxidants is mechanistically connected to the multifactorial etiology of IR, primarily in skeletal muscle tissue, and the subsequent development of T2DM[133]. IR is characterized by the failure of normal insulin levels to regulate glucose homeostasis, which requires much higher insulin levels to properly maintain euglycemic conditions. Although IR is a systemic disease, it occurs locally in insulin-responsive tissues (liver, muscle, fat and pancreas) as well as in the brain and gut, which also participate in glucose homeostasis. Recent data suggest that the endothelium could also be affected by IR, which contributes to the endothelial dysfunction observed in these metabolic derangements. At the cellular level, it is generally accepted that the impairment of insulin signalling in parenchymal cells of insulin-responsive tissues (namely, hepatocytes, adipocytes, myocytes, and β-cells) causes IR in these tissues[134].

Experiments in mice and humans showed that insulin inhibited FABP4 release from adipocytes *in vitro*, consistent with feedback regulation. However, during obesity, FABP4 regulates insulin secretion. Adipocyte IR may impair insulin suppression of FABP4 release, resulting in enhanced FABP4 secretion and increased reciprocal insulin release[80]. Furthermore, *Fabp4*$^{-/-}$ mice showed an increase in body weight but reduced IR in both high-fat diet-induced and genetic obesity mouse models, but the effect of FABP4 on insulin sensitivity was not observed in lean mice[103].

2.5.5. Circulating FABP4

FABP4 has traditionally been implied to act as an intracellular lipid chaperone, mediating the intracellular trafficking and targeting of FA[119]. However, it was shown that extracellular FABP4 has a direct impact on decreasing the contractility of myocardial muscle cells, suggesting that its release into the bloodstream could have direct effects on some peripheral cells and tissues[135] (Fig. 7). FABP4 concentrations in human plasma range from 10 to 50 ng/mL, a level that is much higher than that of several other major adipokines secreted from adipose tissue, such as leptin, TNF-α and IL-6[81]. Plasma levels of FABP4 correlate positively with measures of adiposity (BMI, waist–hip ratio, waist circumference, and fat percentage), suggesting that adipose tissue is the predominant contributor of FABP4 in circulation[136]. Recent data have demonstrated that FABP4 is actively released from differentiated human adipocytes *in vitro* via a non-classical, calcium-dependent mechanism[137-139] responsive to lipolytic activity and the availability of free FAs[138, 139]. FABP4 lacks an N-terminal secretory signal sequence, necessary for the classical secretory pathway (ER/Golgi-dependent pathway). In differentiated human adipocytes, FABP4 secretion is not abolished by blocking the Golgi-dependent secretory pathway *in vitro*, supporting a non-classical secretion mechanism for FABP4. Furthermore, this secretion significantly increased upon treatment of human adipocytes with ionomycin, an ionophore that raises intracellular Ca^{2+} levels[137]. Adipocyte lipase activities, specifically ATGL, and, to a lesser extent, HSL are involved in the regulated secretion of FABP4, and genetic deletion or chemical inhibition of these enzymes dramatically diminishes FABP4 secretion from adipocytes[139]. Lipolysis induces relocalization of FABP4 to a non-classical secretory pathway, which involves the multivesicular body compartment. Once secreted, the majority of FABP4 is detectable in the free form in circulation, but a small fraction persists in vesicles[139].

Thus, FABP4 is actively released by unconventional mechanisms and by adipocyte-derived exosomes and microvesicles from adipocytes by lipolytic signal pathways and/or an intracellular calcium-dependent mechanism[138]. The regulation of FABP4 secretion by nutritional status and lipolysis, and its function in promoting hepatic glucose production and IR, fits well with our current understanding of blood glucose regulation during prolonged fasting, when the release of gluconeogenic substrates (e.g., glycerol) and signals from adipose tissue are synchronized[139]. Adipose tissue becomes resistant to insulin-mediated suppression of lipolysis, which will drive FABP4 release and contribute to increased liver glucose output, leading to hyperglycaemia and T2DM. Hence, although FABP4 carries the properties of a gluco-regulatory hormone of fasting or catabolism, it is paradoxically elevated to signal a "pseudo-fasting" condition in obesity[139]. Furthermore, proteomic analysis showed the presence of FABP4 in cell supernatants derived from differentiated THP-1 macrophages[101], FABP4 is released from human macrophages by a a non-classical secretion mechanism[137].

In the last several years, significant effort has been focused on determining the role of circulating FABP4. Our group and others have shown that FABP4 levels are increased in obesity, MS, T2DM, and familial combined hyperlipidaemia or lipodystrophy syndrome and that these levels are also closely correlated with adverse lipid profiles and IR[136, 140-145]. Furthermore, recent studies have emphasized an important role for circulating FABP4 in the pathogenesis of obesity-related complications, such as T2DM and atherosclerosis[146-148]. Baseline FABP4 serum levels independently predict the risks of developing MS, T2DM and atherosclerotic disease[145, 149-151]. In atherosclerosis, plaque rupture and erosion with ensuing thrombus formation and occlusion of the artery lead to acute clinical complications comprising acute coronary syndrome (ACS), which constitutes one of the leading

causes of death worldwide[152] and the major underlying cause of coronary heart disease[148]. The FABP4 level in serum may serve as a prognostic marker to identify patients at risk for adverse cerebrovascular or cardiovascular events in patients with ACS events[152]. In addition, circulating levels of FABP4 are positively associated with the pathogenesis of NAFLD, which is now recognized as the hepatic manifestation of MS[153]. FABP4 serum levels in polycystic ovary syndrome (PCOS)[154] and obstructive sleep apnea (OSA)[155] patients are greater than those of healthy controls[154]. In PCOS, pretreatment FABP4 levels appear to predict therapeutic long-term response[156]. In OSA, increases in FABP4 expression are associated with its presence and severity[155].

A recent study has found that circulating FABP4 levels are inversely associated with peripheral reactive hyperaemia, a subrogated marker of endothelial dysfunction[157]. In addition, it has been demonstrated that increased levels of FABP4 in atherosclerotic lesions are associated with unstable plaque phenotypes[158, 159]. Furthermore, along with a decrease in eNOS expression, extracellular FABP4 increases the expression of VCAM-1 in addition to E-selectin and leukocyte adhesion to endothelial cells, suggesting that is has a more global effect on endothelial function[128]. It was observed that the amount of intracellular FABP4 increases after FABP4 incubation with respect to non-treated cells, which suggests that FABP4 could be internalized by endothelial cells[128].

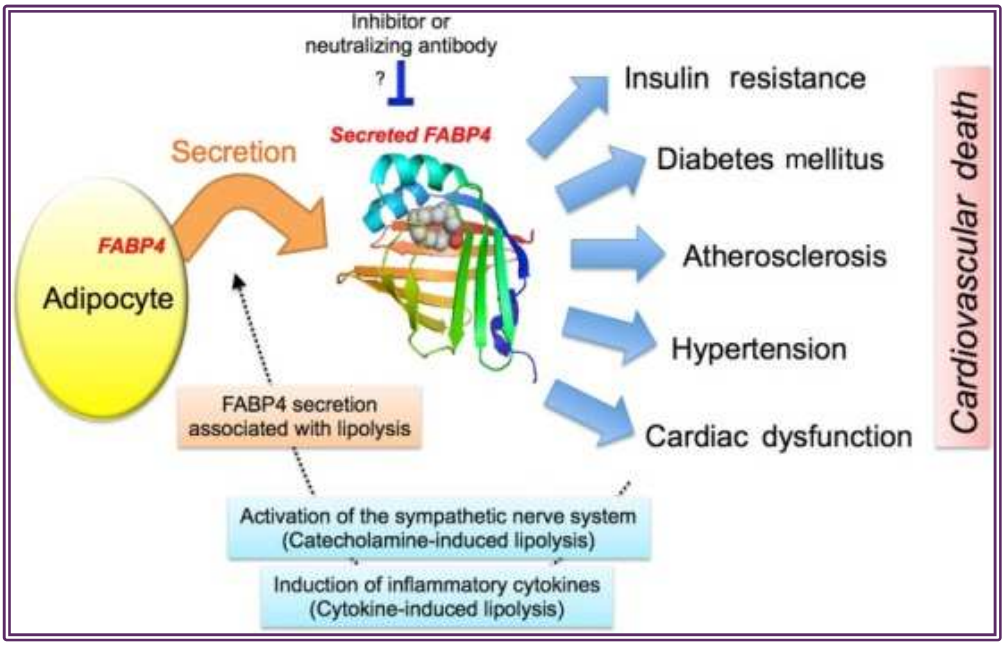

Figure 7. Possible actions of circulating FABP4[101].

2.5.6. FABP4 inhibitors

Inhibition of FABP4 has been proposed as a treatment for T2DM, fatty liver disease and atherosclerosis[106]. Some natural compounds can interfere with FABP4 expression to inhibit it. Resveratrol (3,4',5-trihydroxystilbene), a phytoalexin, is a naturally occurring polyphenolic compound. Many studies using adipocytes have demonstrated that resveratrol has anti-obesity potential by inhibiting preadipocyte differentiation, decreasing adipocyte proliferation, inducing adipocyte apoptosis, decreasing lipogenesis, and promoting lipolysis and FA β-oxidation. The anti-obesity effect of resveratrol in animals seems to be mediated through the stimulation of fat oxidation and metabolism or suppression of adipogenic gene expression, such as *Fabp4*[160]. Green tea catechins and curcumin also own-regulate

the expression of key genes involved in adipocyte differentiation, including *C/EBPα*, *Pparg* and *Fabp4*[160]. Coffee reduced *Pparg* gene expression, as well as other adipocyte marker genes (*Fabp4, LPL, adiponectin*, and *GLUT4*) during adipocyte differentiation in the coffee-treated cells[161].

Sulforaphane is an aliphatic isothiocyanate, and it is converted from glucoraphanin, a major glucosinolate in broccoli and cabbage and is one of the most potent naturally occurring inducers of cytoprotective enzymes. It is known for its antidiabetic properties[162]. Sulforaphane is an inducer of Nrf2 signalling through interruption of the association between Keap1 and Nrf2 that modulates the elevation in NQO1 expression and activity[100]. Pharmacological Nrf2 activation by sulforaphane prevented adipogenesis and lipid accumulation as enhanced Nrf2 activity decreased triglyceride content and tended to decrease free FA content accompanied by decreased expression of FABP4[99] through NQO1 protein[100]. Furthermore, treatment with siphonaxanthin almost completely inhibited mRNA expression of *Fabp4*. Siphonaxanthin is a xanthophyll present in green algae and possesses antiangiogenic and apoptosis-inducing activities and this beneficial effect may result from its inhibitory effects on various kinases, including Akt and ERK[163].

FABP4 displays a naturally low selectivity towards hydrophobic ligands, leading to the possibility of side effects arising from cross-inhibition of other FABP isoforms[164]. Considering that FABP4 comprises approximately 5% of the total adipocyte protein content, inhibitor selectivity might be more important than inhibitor affinity for avoiding cross-inhibition side effects[164]. FABP4 contains essentially one large cavity with a volume of approximately 950 $Å^3$. From crystallographic data of ligands bound to the protein, the interior amino acids are known to specifically bind to endogenous FA. The ubiquity of endogenous FAs in living systems and the high

intracellular concentration of FABP4 (on the order of micromolar) implied that we needed an inhibitor of significantly greater intrinsic potency than endogenous FA[164, 165]. A novel structural class of compounds that bind to FABP4 with significantly greater affinity and FABP isoform selectivity than known endogenous FA substrates was identified; biphenyl azoles in particular are potent (on the order of nanomolar) and selective inhibitors of FABP4. These ligands can serve as useful probes for further investigation of the potential utility of FABP4 inhibitors for the treatment of T2DM, obesity, and atherosclerosis[165]. BMS309403 is an aromatic biphenyl azol compound that exhibits K_i values of <2 nM for both mouse and human FABP4 compared with 250 nM for FABP3 and 350 nM for FABP5[165].

Other synthetic compounds, apart from biphenyl azoles, such as fluorescein, ketazolam, antrafenine, darifenacin, fosaprepitant, paliperidone, risperidone, trovafloxacin, pimozide, levofloxacin and sitagliptin, were identified as inhibitors of FABP4[63, 166]. Fluorescein is extensively used as a fluorescent tracer in diagnostic applications. It is unlikely to be a drug for disease treatment due to serious adverse reactions, including cardiac arrest and anaphylactic shock. Ketazolam, a benzodiazepine derivative, is used for the treatment of anxiety. However, long-term administration of ketazolam results in tolerance and physical dependence. Antrafenine, a phenylpiperazine derivative, is marketed as an analgesic and anti-inflammatory drug, but it is not widely used because it has been replaced by next generation anti-inflammatory drugs. Darifenacin and fosaprepitant are newly approved drugs that are not commercially available. Paliperidone and risperidone are antipsychotic drugs. Trovafloxacin is a broad-spectrum antibiotic that blocks the activity of DNA gyrase and topoisomerase IV in various bacteria[63].

Pimozide is a conventional antipsychotic of the diphenylbutylpiperidine class that has been clinically used for over 30 years[167]. The inhibitory effect of pimozide on

FABP4 elevates the basal protein levels of PPARγ, thereby stimulating adipogenesis in adipocytes and promoting adipocyte differentiation, explaining the weight gain that is frequently observed in patients treated with this drug[167].

Levofloxacin is a broad-spectrum antibiotic used to treat a series of infections, including respiratory tract infections, cellulitis, and urinary tract infections and directly inhibits FABP4 activity in both *in vitro* ligand displacement assays and cell-based functional assays. Furthermore, levofloxacin does not induce adipogenesis in adipocytes, which is the major adverse effect of FABP4 inhibitors[63].

Sitagliptin is a DPP-4 inhibitor that increases glucagon-like peptide 1 (GLP-1) and is used for T2DM treatment[166]. A recent study has demonstrated that sitagliptin decreases serum FABP4 concentration in T2DM patients at least in part via reduction of FABP4 expression and its secretion from adipocytes by direct DPP-4 inhibition[166].

Furthermore, recent studies have investigated the binding of FABP4 towards a group of small molecules structurally related to the non-steroidal anti-inflammatory drug ibuprofen, and it was observed that this binding displayed unexpectedly high affinities with dissociation constants in the nanomolar to micromolar range, which is comparable to those of native FA ligands[162].

MicroRNAs (miRNAs) are endogenous, conserved, non-coding small RNAs that function as post-transcriptional regulators in diverse biological processes[168] and play an important role in tumorigenesis, CVD, T2DM and other diseases. Increasing evidence demonstrated that miRNAs exert a significant effect on adipose tissue development and adipocyte differentiation[169]. miR-135a-5p inhibited preadipocyte differentiation and adipogenesis by impeding the transcriptional induction of

Fabp4 and other adipogenic marker genes, such as *Pparg*, *Cebpa*, and *Fas*[168]. Furthermore, miR-24 significantly inhibited the expression of FABP4[169].

2.5.6.1. BMS309403

BMS309403 (2-(2'-(5-ethyl-3,4-diphenyl-1H-pyrazol-1-yl)biphenyl-3-yloxy) acetic acid) is an aromatic biphenyl azol compound that competes with FAs for the FA-binding pocket of FABP4 with high specificity[50] and competitively inhibits the binding of endogenous FAs[165] (Fig. 8). *In vitro* experiments showed that treatment with BMS309403 resulted in a dose-dependent decrease in FA uptake in wild-type adipocytes[106] and led to markedly reduced triglyceride hydrolase activities in white adipose tissue and adipocyte cell lysates[96]. Cholesterol efflux from human THP-1 macrophages was significantly increased upon treatment with the FABP4 inhibitor coupled with a significant increase in both mRNA and protein levels of the ABCA1 protein, a critical mediator of cholesterol efflux in macrophages. Furthermore, transformation of THP-1 macrophage to foam cells was significantly reduced in the presence of FABP4 inhibitor[106]. In myotubes, BMS309403 significantly increased glucose uptake in a time- and dose-dependent manner, which was paralleled by phosphorylation of AMPKa, p38 and ACC[170]. The inhibition of FABP4 with BMS309403 improved endothelial function in terms of eNOS phosphorylation, NO production and endothelium-dependent relaxation[50].

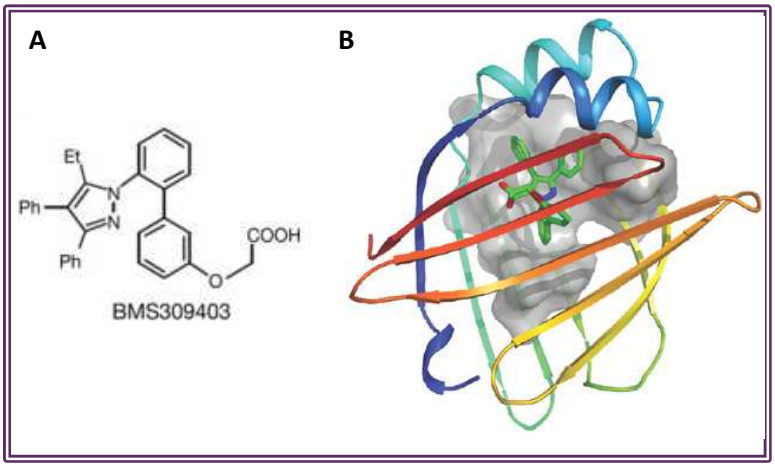

Figure 8. A. Structure of BMS309403[106]. B. Crystal structure of BMS309403 bound to human FABP4[86]

BMS309403 did not influence body weight, systemic glucose or lipid metabolism in *Apoe*$^{-}$/$^{-}$ mice. No significant difference in glucose levels during glucose tolerance tests was observed between the vehicle and FABP4 inhibitor groups[106]**.** In *ob*/*ob* mice, blood glucose levels were decreased after treatment with BMS309403 in the fed and fasted state; free FA levels showed a trend towards an increase, insulin and triglyceride levels increased and adiponectin concentration was decreased. FABP4 inhibitor improves whole body insulin sensitivity through the suppression of hepatic glucose production and enhancement of insulin-stimulated glucose disposal in peripheral tissues[106].

3. Hypothesis and objectives

UNIVERSITAT ROVIRA I VIRGILI
FABP4: INTERACTIONS WITH ENDOTHELIAL CELL PLASMA MEMBRANE AND EFFECTS ON VASCULAR SMOOTH MUSCLE CELLS.
Paula Saavedra García
Dipòsit Legal: T 238-2016

3.1. Hypothesis

FABPs are members of the iLBP family involved in reversibly binding intracellular hydrophobic ligands and trafficking them throughout cellular compartments so they were primarily studied as intracellular proteins. In the last years, multiple FABP isoforms have been detected in the circulation and in general, they are seen as markers of cell injury or death. FABP4 is an adipose tissue-secreted adipokine that is involved in the regulation of energetic metabolism and inflammation. Increased levels of circulating FABP4 have been detected in individuals with cardiovascular risk factors and atherosclerosis .

Vascular dysfunction and migration and proliferation of VSMCs are response to cardiovascular risk factors and precede the development of atherosclerosis. It is demonstrated that increased levels of FABP4 in atherosclerotic lesions are associated with unstable plaque phenotypes and it has a direct effect on peripheral tissues, specifically promoting vascular dysfunction and atherosclerosis; however, the mechanism of action and functions of circulating FABP4 are not clear. Many circulating adipokines interact with peripheral tissues through receptors but if FABP4 has a receptor or not, is unknown. The hypothesis of this study is that circulating FABP4 has a direct effect on peripheral tissues. In particular, at vessel wall level, FABP4 contributes to endothelial dysfunction and artery wall remodelling through interaction with endothelial plasma membrane proteins that act as anchoring elements and/or receptors mediating intracellular signal and/or FABP4 internalization. FABP4 also acts on smooth muscle cells influencing cell migration and proliferation.

3.2. Objectives

1. Study *in vitro* the effect of exogenous FABP4 on endothelial plasma membrane:

    1.1. Determinate the type of interaction between exogenous FABP4 and HUVEC plasma membrane.

    1.2. Identify FABP4 binding proteins on endothelial cell membrane.

2. Determinate the role of FAs in the FABP4/cell membrane interaction mechanism *in vitro*:

    2.1. Study the effect of FABP4 inhibition (BMS309403) on exogenous FABP4 and its interaction with endothelial plasma membrane.

3. Analyze *in vitro* the presence of exogenous FABP4 in HUVEC plasma membrane:

    3.1. Study the interaction between exogenous FABP4 and HUVEC plasma membrane protein at different times.

    3.2. Study exogenous FABP4 internalization in HUVECs.

4. Study *in vitro* the effect of exogenous FABP4 on peripheral tissues (artery, liver, macrophages and smooth muscle).

5. Analyze *in vitro* the effect of exogenous FABP4 on migration and proliferation in human coronary artery smooth muscle cells (HCASMCs).

    5.1. Study the migration in HCASMCs in presence of exogenous FABP4.

    5.2. Study the proliferation in HCASMCs in presence of exogenous FABP4.

    5.3. Determinate the effect of FABP4 on ERK1/2 and Akt pathways.

        5.3.1. Study the effect of FABP4 on ERK1/2 and Akt activation.

5.3.2. Study the effect of FABP4 on the expression of transcription factor activated by the MAPK signal transduction.

5.3.3. Study the effect of FABP4 on the expression of proteins involved in cell cycle regulation and cell migration.

4. Papers

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New insights into circulating FABP4: Interaction with cytokeratin 1 on endothelial cell membranes

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ABSTRACT

Fatty acid-binding protein 4 (FABP4) is an adipose tissue-secreted adipokine that is involved in the regulation of energetic metabolism and inflammation. Increased levels of circulating FABP4 have been detected in individuals with cardiovascular risk factors. Recent studies have demonstrated that FABP4 has a direct effect on peripheral tissues, specifically promoting vascular dysfunction; however, its mechanism of action is unknown. The objective of this work was to assess the specific interactions between exogenous FABP4 and the plasma membranes of endothelial cells. Immunofluorescence assays showed that exogenous FABP4 localized along the plasma membranes of human umbilical vein endothelial cells (HUVECs), interacting specifically with plasma membrane proteins. Anti-FABP4 immunoblotting revealed two covalent protein complexes containing FABP4 and its putative receptor; these complexes were approximately 108 kDa and 77 kDa in size. Proteomics and mass spectrometry experiments revealed that cytokeratin 1 (CK1) was the FABP4-binding protein. An anti-CK1 immunoblot confirmed the presence of CK1. FABP4-CK1 complexes were also detected in HAECs, HCASMCs, HepG2 cells and THP-1 cells. Pharmacological FABP4 inhibition by BMS309403 results in a slight decrease in the formation of these complexes, indicating that fatty acids may play a role in FABP4 functionality. In addition, we demonstrated that exogenous FABP4 crosses the plasma membrane to enter the cytoplasm and nucleus in HUVECs. These findings indicate that exogenous FABP4 interacts with plasma membrane proteins, specifically CK1. These data contribute to our current knowledge regarding the mechanism of action of circulating FABP4.

1. Introduction

Fatty acid-binding protein 4 (FABP4; adipocyte-FABP; aP2) is a member of the family of intracellular fatty acid-binding proteins (FABPs), which are ~15 kDa in weight and 126–134 amino acids in length [1,2]. FABPs are expressed abundantly (1–5% of cytosolic proteins) in cells involved in active lipid metabolism. Members of the FABP family exhibit unique tissue-specific expression patterns and are named according to the tissues in which they were first identified [3]. FABPs are capable of binding a variety of hydrophobic ligands, such as long-chain fatty acids, eicosanoids, leukotrienes and prostaglandins [4–6]. However, the divergent sequences of the members confer subtle differences in their ligand-binding properties and may also indicate different protein–protein interaction partners depending on the cellular context. Indeed, it has been shown that intracellular adipocyte-, epithelial-, and heart-type FABPs interact with hormone-sensitive lipase, whereas the intestinal and liver isoforms do not [3–7]. It is also known that FABP4 interacts with Janus Kinase 2 in a fatty acid-dependent manner, establishing a new role for FABP4 as a fatty acid sensor that affects cellular metabolism via protein–protein interactions [8]. FABP4 is highly expressed in adipocytes and macrophages and has a role in lipid metabolism in both cell types [8]. FABP4 expression has also been reported in endothelial cells [9]. The biological relevance of FABP4 is underscored by the findings that FABP4 knockout (FABP4 $^{-/-}$) mice exhibit marked protection against insulin resistance, atherosclerosis, fatty liver disease, and asthma [5,10–14]. Consistent with these studies, a small-molecule inhibitor of FABP4 (BMS309403) has been found to be an effective therapeutic agent for the treatment of atherosclerosis and type 2 diabetes mellitus (T2DM) in mouse models [15]. FABP4 is secreted from adipocytes via a non-classical, calcium-dependent mechanism [16] that is regulated by lipolytic pathways [17]. It has been demonstrated that adipocyte lipases, specifically adipose triglyceride lipase (and, to

Abbreviations: CK1, cytokeratin 1; FABP4, fatty acid-binding protein 4; FABP4-His, polyhistidine-tagged fatty acid-binding protein 4; FABP, fatty acid-binding proteins; T2DM, type 2 diabetes mellitus; NO, nitric oxide

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a lesser extent, hormone-sensitive lipase), are involved in the regulated secretion of FABP4 [18]. In the last several years, much effort has been focused on determining the role of circulating FABP4. We and other authors have shown that FABP4 levels are increased in obesity, metabolic syndrome, T2DM, and familial combined hyperlipidemia and lipodystrophy syndromes. In addition, FABP4 levels are closely correlated with adverse lipid profiles and insulin resistance [19–25]. FABP4 serum levels independently predict the risks of developing metabolic syndrome, T2DM and atherosclerotic disease [24,26–28]. A recent study has found that circulating FABP4 levels are inversely associated with peripheral reactive hyperemia, a surrogate marker of endothelial dysfunction [29]. In addition, it has been demonstrated that increased levels of FABP4 in atherosclerotic lesions are associated with unstable plaque phenotypes [30,31]. Extracellular FABP4 also directly decreases the contractility of myocardial muscle cells, suggesting that its release into the bloodstream could directly affect certain peripheral cells and tissues [32]. Recent results from our group have shown that along with a decrease in eNOS expression, extracellular FABP4 increases the expression of vascular cell adhesion protein 1 (VCAM1), E-selectin and leukocyte adhesion to endothelial cells; these results suggest that FABP4 has a global effect on endothelial function [33]. Furthermore, FABP4 directly affects the migration and proliferation of human artery coronary smooth muscle cells (HCASMCs). This result suggests a role for FABP4 in vascular remodeling that is mediated primarily through a MAPK-dependent pathway, which activates the transcription factors c-jun and c-myc in HCASMCs [34].

These data prompted us to investigate the interactions of exogenous FABP4 with plasma membrane proteins in endothelial cells. Here, based on the results of immunofluorescence and cross-linking immunoblot experiments, we report the cell-surface localization of exogenous FABP4 protein complexes. Proteomics analysis subsequently revealed that FABP4 binds specifically with cytokeratin 1 (CK1).

2. Materials and methods

2.1. Cell culture, treatment and protein extracts

Human umbilical vein endothelial cells (HUVECs) (passage 3) and human aortic endothelial cells (HAECs) were cultured in Medium 200 supplemented with 2% low serum growth supplement (LSGS) and 1% gentamicin/amphotericin (GIBCO®, Oregon, OR, USA). Human coronary aortic smooth muscle cells (HCASMCs) were cultured in Medium 231 supplemented with 2% smooth muscle growth supplement (SMGS) and 1% gentamicin/amphotericin (GIBCO®). Monocyte-derived THP-1 macrophages (DSMZ, Braunschweig, Germany) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (BioWest, Kansas City, MO, USA). HepG2 cells (ATTC, Manassas, VA, USA) were cultured in MEM medium supplemented with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine and 1% non-essential amino acids (NEAA) (BioWest).

The cells were incubated with or without human recombinant FABP4 (100 ng/mL) (BioVendor, Heidelberg, Germany) or human recombinant polyhistidine-tag FABP4 (FABP4-His) (Enzo Life Sciences, San Diego, CA, USA) for the indicated times. In some experiments, HUVECs were incubated with the FABP4 inhibitor BMS309403 {2-[2′-(5-ethyl-3,4-diphenyl-1H-pyrazol-1-yl)biphenyl-3-yloxy] acetic acid} at 10 μM (Calbiochem, San Diego, CA, USA). Dimethyl sulfoxide (DMSO) was used as a solvent control (Sigma-Aldrich).

Cell plasma membrane lysates were obtained as previously reported [35]. Briefly, the cells were incubated with cold buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM Ca^{2+}, 1% Triton X-100, 1% NP40, and protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and kept on ice for 15 min. After incubation, the lysates were centrifuged at 16,000 × g for 15 min, and the supernatant was collected. Nuclear and cytoplasmic protein extracts were obtained as previously described [34].

2.2. Immunoblotting

Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Electrophoresis and immunoblot analyses were performed using the NuPAGE protein analysis system (Invitrogen Life Technologies, Carlsbad, CA, USA). The membranes were incubated with anti-FABP4 (R&D Systems, Minneapolis, MN, USA), anti-His-tag (Abcam, Cambridge, MA, USA), anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-CD31 (DAKO, Glostrup, Denmark) and anti-cytokeratin 1 (CBL266) (Merck Millipore, Billerica, MA, USA) antibodies. The antigen-antibody complexes were detected by incubating the membrane with HRP-conjugated anti-IgG antibodies (DAKO). The bands were visualized using a ChemiDoc Image System and quantified with Image Lab analysis software (Bio-Rad). The relative levels of FABP4 were quantified after normalization with CD31 or actin levels. All of the values were expressed in arbitrary units (AU). The molecular weights of the bands were assigned by comparing the band weights with BenchMark™ Protein Ladder (Invitrogen Life Technologies) using ImageQuant TL v7.0 v (GE Healthcare, Wauwatosa, WI, USA).

2.3. Immunofluorescence microscopy

For the colocalization experiments, HUVECs were incubated in chambered slides (Nunc, Roskilde, Denmark) with or without FABP4, fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at 4 °C and washed at each step three times with DPBS (GIBCO®) for 5 min. The cells were then incubated overnight at 4 °C with anti-FABP4 and anti-CD31 antibodies and further incubated with Alexa Fluor® 488 anti-goat and Alexa Fluor® 532 anti-mouse antibodies (Invitrogen Life Technologies) for 3 h at RT. The antibodies were diluted in the blocking solution (DPBS, 2% FBS, and 0.1% BSA). Confocal immunofluorescence images were captured with a Nikon Eclipse TE2000-E microscope and processed with EZ-C1 3.40 software (Nikon, Chiyoda, Tokyo, Japan).

For the internalization experiments, the cells were permeabilized after fixation with a solution containing DPBS, 2% FBS, 0.1% BSA, and 0.1% Triton X-100 for 5 min at RT. The cells were then incubated with blocking solution for 20 min at RT. The cells were incubated overnight at 4 °C with an anti-FABP4 antibody and further incubated with an Alexa Fluor® 488 anti-goat antibody for 3 h at RT. The cells were then incubated with the nuclear stain DAPI (4′,6-diamidino-2-phenylindole, dilactate) and CellMask™ Deep Red Plasma Membrane Stain (Invitrogen Life Technologies) for 5 min. Immunofluorescence images were captured with an Olympus IX71 inverted microscope, processed with Cell^F Software (Olympus, Shinjuku-ku, Tokyo, Japan) and quantified with ImageJ (Fiji, Madison, WI, USA).

2.4. HUVEC crosslinking

After incubation with or without FABP4-His, the HUVECs were cross-linked with 2% formaldehyde in DPBS at RT for 30 min, as previously reported [36,37]. The cells were washed once in DPBS, and HUVEC plasma membrane protein extraction was performed as mentioned above.

2.5. Polyhistidine-tagged protein purification

Plasma membrane proteins from HUVECs that were incubated with or without FABP4-His were purified according to the instructions provided with the HisPur™ Cobalt Purification Kit, which was purchased from Pierce (Rockford, IL, USA).

2.6. Ligand screening assay

Plasma membrane proteins from HUVECs incubated with or without FABP4-His were analyzed with the FABP4 Inhibitor/Ligand Screening Assay Kit (Cayman Chemical Company, Ann Harbor, MI, USA) to detect

FABP4 ligands. Briefly, the protein lysates were incubated with a fluorescent probe that fluoresces when bound to FABP4 and recombinant FABP4 (10 mM). Any FABP4 ligand can displace the fluorescent probe, thereby reducing fluorescence. We used arachidonic acid (1.28 mM), a known ligand of FABP4, as a positive control. The binding of the fluorescent probe to FABP4 was monitored by measuring excitation at 370 mm and emission at 475 mm using a Synergy HT fluorometer (BioTek, Winooski, VT, USA). The obtained results were presented as the percent fluorescence of each sample (plasma membrane proteins) relative to the fluorescence maximum (FABP4 + fluorescence probe).

2.7. Small interfering RNA (siRNA) transfection

To knock down FABP4 expression, ON-TARGETplus siRNAs targeting human FABP4 (J-008,853–08–0005) and GAPDH (D-001,830–01–05) as a control were purchased from Dharmacon (Lafayette, CO, USA). The cells were transiently transfected using Lipofectamine® RNAiMax (Invitrogen Life Technologies) and analyzed after 72 h.

2.8. Quantitative real-time PCR

Total RNA was isolated from the cells using the ABI PRISM 6100 Nucleic Acid PrepStation kit (Applied Biosystems, Carlsbad, CA, USA). The absorbance at 260 nm was used to determine the RNA concentration, and the 260/280 ratio was used to determine the RNA quality. Total RNA (0.5 μg) was reverse transcribed to cDNA using random hexamers and SuperScript II (Invitrogen Life Technologies), following the manufacturer's protocol. Pre-designed and validated TaqMan Gene Expression Assay (Life Technologies) primers and probes were obtained for FABP4 (Hs00609791_m1) and 18S (Hs99999901_s1) and used for real-time PCR amplification. The mRNA expression of each gene and sample was calculated using the recommended 2-ΔΔCt method. Untreated cells were considered as controls in this experiment. 18S was used as a housekeeping gene to normalize the results for the gene of interest.

2.9. Proteomics sample preparation and MS analysis

Concentrated HUVEC plasma membrane proteins were incubated with or without FABP4-His, and impurities were removed using a 2D clean-up kit (GE Healthcare) according to the manufacturer's instructions. After quantification, the samples were separately labeled with either Cy3 or Cy5 according to the manufacturer's protocol. The labeled samples were mixed and combined for MS analysis.

First-dimension separation was performed with an IPGphor isoelectric focusing (IEF) unit (GE Healthcare), in which the samples were loaded onto 7-cm, pH 3–10 immobilized pH gradient (IPG) strips with passive rehydration for 16 h. This step was followed by isoelectric focusing for a total of 7000 V/h. The strips were immediately equilibrated in SDS equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 75 mM Tris Cl, pH 8.8, and 0.005% bromophenol blue) for 15 min with 1% (*w*/*v*) DTT. The strips were then equilibrated for 15 min with SDS buffer with 2.5% (*w*/*v*) iodoacetamide (IAA). For the second dimension of separation, the strips were applied directly to a 12% SDS-polyacrylamide gel. Immediately after 2D-DIGE was performed, the gels were scanned with a Pharos FX™ Plus Molecular Imager scanner using excitation/emission filters of 532/580 nm for Cy3 and 633/670 nm for Cy5 to generate multiplexed DIGE image files. Statistical and quantitative analyses of the changes in the spots on the images were performed using Progenesis SameSpots software v4.1 (Nonlinear Dynamics). Spots that were only present in the FABP4-His-incubated cells were selected for further identification. Lastly, the gel was stained with a PlusOne Silver Staining Kit (GE Healthcare), following the manufacturer's recommendations.

Protein spots were selected and digested with trypsin [38]. Briefly, the gel pieces were de-stained by three washes in 25 mM NH_4HCO_3, with vortexing, for 15 min and one wash in 100% CH_3CN. The gel pieces were dried and then swelled by incubation with digestion buffer (50 mM NH_4HCO_3 and 12.5 ng/mL of autolysis-stabilized trypsin) (Promega, Madison, WI, USA) at 37 °C for 16 h. The peptides were extracted sequentially in 0.1% trifluoroacetic acid (TFA) and 0.1% TFA in 50% CH_3CN and vacuum-dried. Lastly, the peptides were washed using Zip-tip C18 columns (Millipore), and the elutions were recovered with a 50% CH_3CN and 0.1% TFA solution.

The peptides were spotted onto an AnchorChip (Bruker, Bremen, Germany) target using α-cyano-4-hydroxy-cinnamic acid as a matrix. The peptides were then analyzed with a MALDI TOF/TOF (Ultraflextreme, Bruker Daltonics) instrument operated in the positive ion mode. The analyzed mass range was 700–3500 Da, with ion suppression of up to 600 Da. MS and MS/MS analyses were performed automatically. For the MS analysis, 1500 single-shot spectra were accumulated by recording 50-shot spectra at 10 random positions using fixed laser attenuation. The selection of precursor ions for MS/MS was performed using an Autoexecute workflow with FlexControl software v3.4 (Bruker Daltonics). For the MS/MS analysis, 100 single-shot spectra were recorded for precursors, and 3000 single-shot spectra were recorded for the fragment ion spectra. The peptide and tandem mass spectra were searched using MASCOT (Matrix Science Inc., MA) against the Swiss-Prot database (released on 03/2013), focusing on human taxonomy. The search parameters were as follows: MS accuracy = 75 ppm, MS/MS accuracy = 0.7 Da, two missed cleavages by trypsin allowed, carbamidomethyl of cysteine as a fixed modification and oxidation of methionine as a variable modification.

2.10. GeLC-MS/MS analysis

The concentrations of plasma membrane proteins from HUVECs that were incubated with or without FABP4-His were measured by the Bradford assay, using BSA as a standard. SDS-PAGE analysis was carried out with 5 μg of these samples, which were run on a 12% acrylamide minigel. After completion of the run, the gel was visualized by colloidal Coomassie Blue stain (Invitrogen Life Technologies). Following visualization of the gels, each lane was completely cut out in ten pieces using a scalpel and placed into a 0.5-mL Eppendorf tube for in-gel digestion.

The protein bands were excised from the gel and shrunk by dehydration in acetonitrile, which was then removed. The resulting spots were dried in a vacuum centrifuge. The gel pieces were covered with 10 mM dithiothreitol (DTT), and the proteins were reduced for 1 h at 56 °C. After cooling to RT, the DTT solution was replaced with approximately the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3. After 45 min of incubation at an ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 100 mM NH_4HCO_3 for 10 min, dehydrated by the addition of acetonitrile, swelled by rehydration in 100 mM NH_4HCO_3, and shrunk again by addition of the same volume of acetonitrile. The liquid phase was removed, and the gel pieces were completely dried in a vacuum centrifuge. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/mL of autolysis-stabilized trypsin (Promega) in an ice-cold bath. After 45 min, the supernatant was removed and replaced with the same buffer without trypsin to keep the gel pieces wet during enzymatic cleavage (37 °C, overnight). The peptides were extracted by one change of 20 mM NH_4HCO_3 and three changes of 5% formic acid in 50% acetonitrile (20 min for each change) at RT and dried.

Analysis was performed with an Esquire HCT ion trap mass spectrometer (Bruker) coupled to a nano-HPLC system (Proxeon, Denmark). The samples were first concentrated on a 300-μm i.d. 1-mm PepMap nanotrapping column and then loaded onto a 75-μm i.d. 15-cm PepMap nanoseparation column (LC Packings, The Netherlands). The peptides were eluted with an acetonitrile gradient (gradient: 0–60% B in 60 min; B = 80% acetonitrile and 0.1% formic acid in water; flow rate = 300 nL/min) through a PicoTip emitter nano-spray needle (New Objective, Woburn, MA, USA) onto the nanospray ionization

source of the ion trap mass spectrometer. MS/MS fragmentation (1.9 s, m/z 100–2800) was performed for two of the most intense ions, as determined from a 1.2-s MS survey scan (m/z 310–1500), using a dynamic exclusion time of 1.2 min for precursor selection. An automated optimization of the MS/MS fragmentation amplitude starting from 0.60 V was used. The proteins were identified by searching the UniProt-SwissProt 57.9 human database using Mascot (Matrix Science, London, UK). The MS/MS spectra were searched with a precursor mass tolerance of 1.5 Da, fragment tolerance of 0.5 Da, trypsin specificity with a maximum of two missed cleavages allowed, carbamidomethylation as a fixed modification, and methionine oxidation as a variable modification. The positive identification criterion was an individual Mascot score for each peptide MS/MS spectrum that was higher than the corresponding homology threshold score.

2.11. Statistical analysis

The results are the average of 3 independent experiments, which were each performed in duplicate (presented as the mean ± SEM). GraphPad Prism was used for statistical analysis (version 5, San Diego, CA, USA), and Student's t-test was used to compare the means. Differences between the two groups were considered statistically significant at $p < 0.05$.

3. Results

3.1. Exogenous FABP4 is found in plasma membranes of HUVECs

We incubated HUVECs with or without FABP4-His for 5 min, 15 min, 30 min, 1 h, 2 h and 4 h, and the plasma membrane protein extracts were analyzed. An anti-FABP4 immunoblot revealed a 2.2-fold increase in FABP4 levels when the cells were incubated with FABP4-His for 5 min compared with the cells incubated without FABP4-His ($p < 0.05$). When cells were incubated without exogenous FABP4, the levels remained constant throughout the 4 h of the experiment (Fig. 1A).

HUVECs were incubated with or without exogenous FABP4 for 5 min, and double-immunofluorescence staining with anti-FABP4 and anti-CD31 (a HUVEC plasma membrane protein marker) without permeabilization was performed. Confocal images revealed colocalization of FABP4 and CD31 (Fig. 1B). Immunofluorescence images showed an increased presence of FABP4 in cells that were incubated with exogenous FABP4 compared to those that were not (1.3-fold increase, $p < 0.05$) (Fig. 1C).

After knocking down FABP4 expression with siRNA, we repeated the immunofluorescence experiments without permeabilization and observed an increase in the amount of FABP4 in HUVECs incubated with exogenous FABP4 (2.3-fold increase, $p < 0.05$) (Fig. 1C).

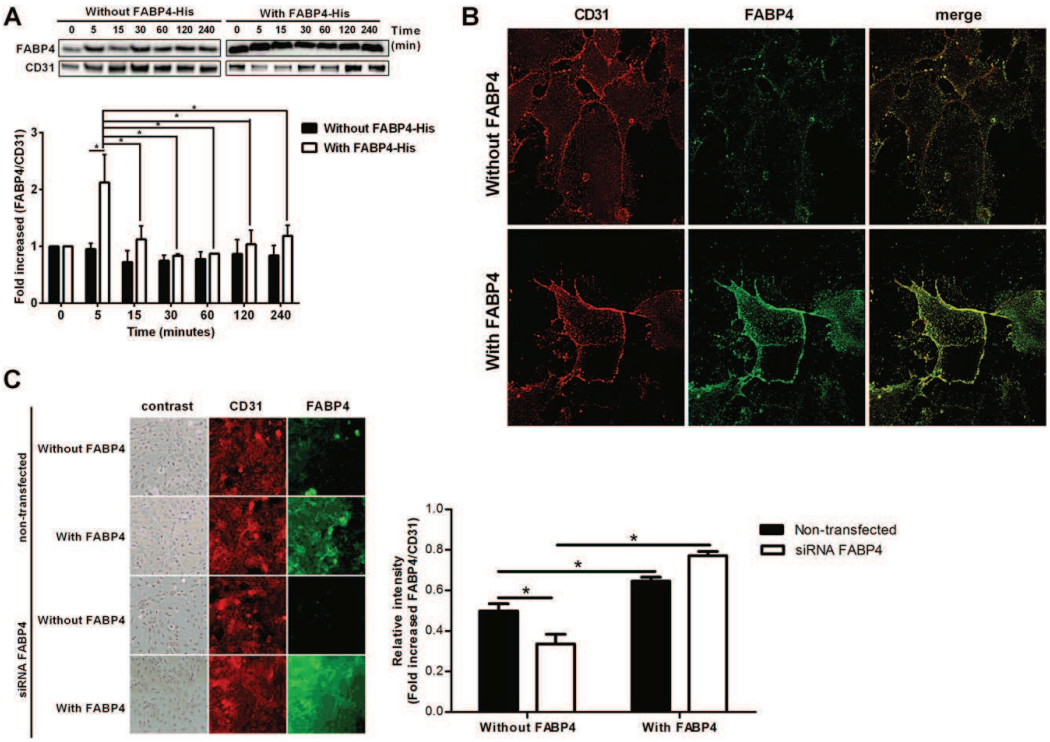

Fig. 1. The presence of exogenous FABP4 in the plasma membranes of HUVECs. (A) FABP4 and CD31 western blots of plasma membrane protein lysates from HUVECs that were incubated with or without exogenous FABP4 (100 ng/mL) for different times (0–4 h). The bar graph represents the quantitation of FABP4 expression levels in the plasma membrane. (B) Confocal images from double immunofluorescence staining for CD31 (Alexa Fluor® 532 dye) and FABP4 (Alexa Fluor® 488 dye), without plasma membrane permeabilization. The cells were incubated with or without exogenous FABP4 for 5 min. (C) Double immunofluorescence staining for CD31 (Alexa Fluor® 532 dye) and FABP4 (Alexa Fluor® 488 dye) was performed without membrane permeabilization in both non-transfected and FABP4-siRNA-transfected HUVECs that were incubated with or without exogenous FABP4 for 5 min. The quantification of FABP4 was performed after normalization for CD31 levels. The results are presented as the mean ± SEM and represent the average of 3 independent experiments (*$p < 0.05$).

3.2. Exogenous FABP4 interacts specifically with plasma membrane proteins

We performed a ligand-screening assay using plasma membrane proteins from HUVECs to determine whether the proteins present in the extracts were bound to FABP4. We analyzed increasing protein concentrations (from 0 to 60,000 pg/mL) of plasma membrane proteins from HUVECs incubated with or without exogenous FABP4 (100 ng/mL) for 5 min. We observed that when cells were incubated with exogenous FABP4, the amount of FABP4 protein detected by fluorescence increased in a concentration-dependent manner (Fig. 2A). This increase was presumably due to the presence of exogenous FABP4 in the plasma membrane. However, when cells were incubated without exogenous FABP4, we observed a concentration-dependent reduction in fluorescence, indicating the presence of FABP4 ligands in the plasma membranes of the HUVECs (Fig. 2A).

Next, HUVECs were incubated with or without FABP4-His (100 ng/mL) for 5 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-Tag purification and western blotting. An anti-FABP4 western blot showed no bands for the cells incubated without FABP4-His, and 4 different bands (approximately 16, 33, 77 and 108 kDa) were detected for the cells incubated with FABP4-His (Fig. 2B and Supp. Fig. 1S). An anti-6X His-tag western blot also showed the 4-band profile (Fig. 2C). HUVECs that were incubated with high levels of tag-free FABP4 (100 ng/mL) and low levels of FABP4-His (10 ng/mL) showed only 16- and 33-kDa bands (the 33-kDa band was a FABP4 homodimer [39]), indicating that the bands at 77 and 108 kDa represented specific complexes formed by exogenous FABP4 and its putative receptor (Fig. 2B and Suppl. Fig. 1S).

We incubated HUVEC plasma membrane proteins in the presence or absence of FABP4-His inside cobalt columns to confirm complex formation. Anti-FABP4 and anti-6X His-tag immunoblots showed 16-, 33-, 77- and 108-kDa bands, confirming the existence of these complexes (Fig. 2D).

We next knocked down FABP4 expression in HUVECs (Figs. 1C, 2E) and incubated the cells with or without FABP4-His (100 ng/mL) for 5 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-tag purification and western blotting. An anti-FABP4 western blot (Fig. 2F) showed 4 different bands (approximately 15, 33, 77 and 108 kDa) in the FABP4-deficient cells incubated with FABP4.

HUVECs were incubated with or without FABP4-His (100 ng/mL) for 5 min, 10 min or 15 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-Tag purification and western blotting to determine whether the interaction between FABP4 and its putative receptor was stable or transient. We observed that the complex formation peaked at 5 min (Fig. 3A); the amount of the complex observed at the plasma membrane began to decrease after 5 min.

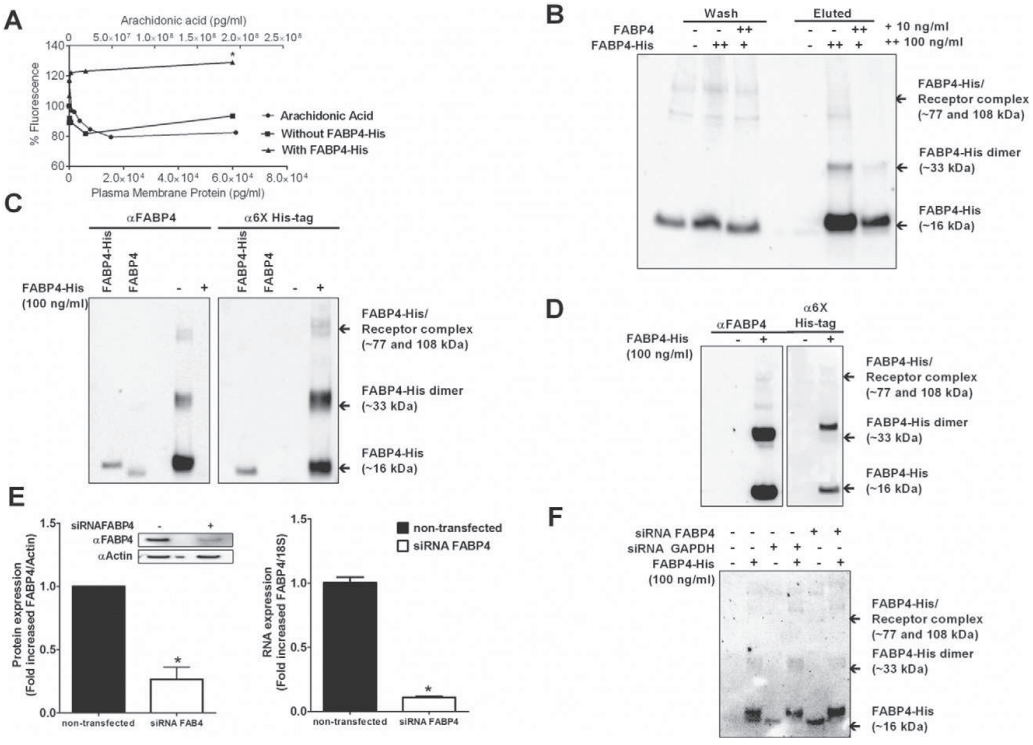

Fig. 2. Specific interactions between exogenous FABP4 and plasma membrane proteins from HUVECs. (A) Displacement curves of plasma membrane proteins from HUVECs incubated with or without FABP4-His (100 ng/mL); arachidonic acid (1.28 mM) was included as a positive control. (B) Western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His and after poly-His-tag purification (eluted and wash fractions), using antibodies against FABP4. (C) Western blot of FABP4-His recombinant protein, FABP4 recombinant protein and plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His and after poly-His-tag purification, using antibodies against FABP4 and the 6X His-tag. (D) Western blot of plasma membrane proteins incubated in cobalt resin columns with or without FABP4-His and visualized with anti-FABP4 and anti-6X His-tag antibodies. (E) FABP4 expression after transfecting HUVECs with siRNA FABP4 as determined by western blot analysis and quantitative real-time PCR. (F) FABP4 western blot of plasma membrane protein lysates from non-transfected and FABP4- and GAPDH-siRNA-transfected HUVECs that were incubated with or without FABP4-His and subjected to poly-His-tag purification. The results are presented as the mean ± SEM and represent the average of 3 independent experiments ($^*p < 0.05$).

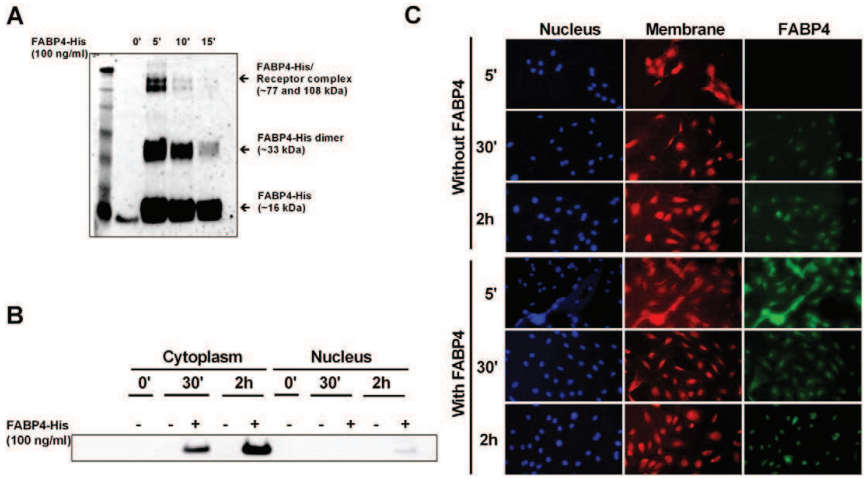

Fig. 3. Exogenous FABP4 is internalized by HUVECs. (A) FABP4 western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4 (100 ng/mL) for different times (0–15 min) and poly-His-tag purification. (B) Western blot, using antibodies against FABP4, of cytoplasmic and nuclear protein lysates from HUVECs that were incubated with or without FABP4-His (100 ng/mL) for different times (0–2 h) and subjected to poly-His tag purification. (C) Triple immunofluorescence staining of the nucleus (DAPI), membrane (CellMask) and FABP4 (Alexa Fluor® 488 dye) was performed on permeabilized HUVECs that were incubated with or without exogenous FABP4 for three different periods of time (5 min, 30 min or 2 h).

To investigate whether exogenous FABP4-His crosses the plasma membrane to enter the cytoplasm and nucleus in HUVECs, we incubated cells with or without FABP4-His for different times (0 min, 30 min or 2 h) and extracted the cytoplasmic and nuclear proteins. Following poly-His-tag purification, immunoblot analysis revealed the presence of FABP4 in the cytoplasm after incubation with exogenous FABP4 for 30 min or 2 h and the presence of FABP4 in the nucleus after incubation with exogenous FABP4 for 2 h (Fig. 3B). After permeabilizing the cells, triple fluorescence staining with anti-FABP4, CellMask and DAPI was performed on HUVECs that had been incubated with or without exogenous FABP4 for 5 min, 30 min or 2 h. In cells that were incubated with exogenous FABP4, the FABP4 immunofluorescence (green) changed from being associated with the plasma membrane (5 min) to being associated with the nucleus (2 h), which indicated that FABP4 migrated from the plasma membrane to the nucleus (Fig. 3C). Conversely, in cells that were not incubated with exogenous FABP4, the same immunofluorescence profile did not appear (Fig. 3C). These data were corroborated by western blot (Fig. 3B).

3.3. Fatty acids are required for the formation of FABP4 protein complexes

HUVECs were incubated with or without FABP4-His (100 ng/mL) in the presence or absence of the inhibitor BMS309403 (10 μM) for 5 min. This incubation was followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-tag purification and western blotting. Fig. 4 indicates weak decreases in the formation of the 108 and 77 kDa complexes, suggesting that the binding of fatty acids to FABP4 was necessary for these interactions.

3.4. Identification of cytokeratin 1 as the binding protein

Two-dimensional gel electrophoresis was performed with plasma membrane extracts from HUVECs that were incubated with or without FABP4-His followed by formaldehyde cross-linking, plasma membrane protein extraction and poly-His-tag purification (Fig. 5A). The samples were labeled with different CyDye fluorochromes (Cy5 for proteins

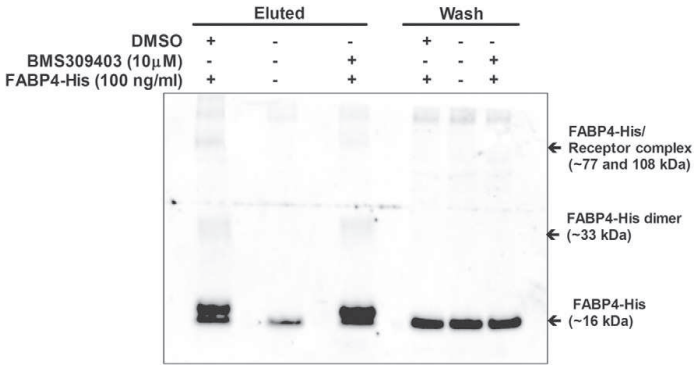

Fig. 4. BMS309403 decreases exogenous FABP4 complex formation. FABP4 western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His (100 ng/mL), with or without BMS309403 (10 μM), and following poly-His tag purification (eluted and wash fractions).

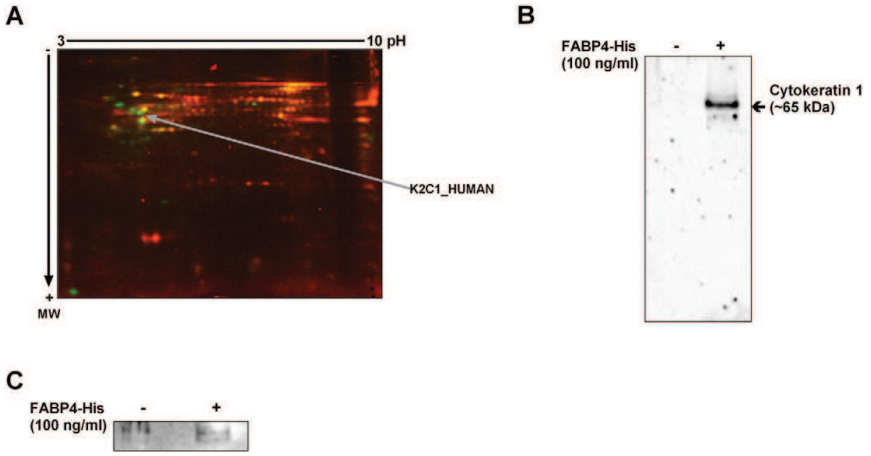

Fig. 5. Identification of CK1 as a FABP4 binding protein. (A) Representative overlapping 2D-GEL expression maps of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His (100 ng/mL) and following labeling with fluorescent dyes: Cy3 (green) corresponds to incubation with FABP4, and Cy5 (red) corresponds to incubation without FABP4. This image is representative of 1 of 3 analyzed gels. (B) CK1 western blot of plasma membrane protein lysates from HUVECs after incubation with or without FABP4-His and poly-His tag purification. (C) Western blot of plasma membrane proteins that were incubated in cobalt resin columns with or without FABP4-His and visualized with antibodies against CK1.

from HUVECs incubated without FABP4-His and Cy3 for proteins from HUVECs incubated with FABP4-His). A total of 98 spots were detected, 82 of which were detected in the samples incubated both with and without exogenous FABP4 and 16 that were only present in the sample incubated with exogenous FABP4. These 16 spots were excised, trypsin-digested and analyzed by MALDI-TOF/TOF. The mass spectrometry results showed a compatible binding profile between FABP4 and cytokeratins, specifically human CK1 (P04264 (K2C1_HUMAN)). This identification was confirmed by another proteomic strategy, GeLC-MS/MS analysis, for which protein samples were fractioned by performing 1D-gel electrophoresis prior to nano-LC ESI. Each lane was cut into 10 small pieces, in-gel digestion was performed, and the peptides were analyzed with a nano-LC ESI q-TOF mass spectrometer. Lastly, definitive validation was achieved by western blot using an anti-CK1 antibody (Fig. 5B). The immunoblot revealed three bands of approximately 108, 77 and 65 kDa. The 108- and 77-kDa complexes were formed by FABP4 and CK1, while the 65-kDa band represented CK1 released from the complexes in the western blot.

3.5. Exogenous FABP4 forms protein complexes in HAECs, HCASMCs, HepG2 cells and THP-1 cells

HAECs, HCASMCs, HepG2 cells and THP-1 cells were incubated with or without FABP4-His (100 ng/mL) for 5 min, followed by formaldehyde cross-linking, plasma membrane protein extraction, poly-His-tag purification and western blotting. The anti-FABP4 immunoblot (Fig. 6A) showed the 4-band profile (15, 33, 77 and 108 kDa) for these cells, indicating that FABP4 formed protein complexes with plasma membrane proteins. The anti-CK1 immunoblot (Fig. 6B) also indicated that these protein complexes were formed by FABP4 and CK1.

4. Discussion

The major finding of this study is that exogenous FABP4 is able to interact with plasma membrane proteins, forming specific protein complexes with CK1 in HUVECs. We demonstrated that exogenous FABP4 is localized in the plasma membrane in specific protein complexes in HUVECs and that fatty acids are required to form FABP4 protein complexes.

Previous studies have postulated that FABP4 is a plasmatic biomarker of metabolic syndrome, T2DM and atherosclerotic disease [24,26–28]. In this study, interactions between exogenous FABP4 and the plasma membranes of HUVECs were shown, supporting the hypothesis that circulating FABP4 is not simply a biomarker but that it may have a causal effect by interacting with peripheral cells. In addition, we observed that these interactions occurred through specific protein complexes, even in FABP4-deficient HUVECs.

We observed that exogenous FABP4 could be internalized into HUVECs, passing through the cell membrane into the cytoplasm after 30 min and into the nucleus after 2 h. It is known that CK1 is an endothelial receptor that aids in the internalization of myeloperoxidase [40], so it is possible that CK1 also aids in the internalization of FABP4. However, it is not known whether this internalization occurs due to the interaction between FABP4 and CK1 or whether free FABP4 (15 kDa) can pass through the membrane into the cytoplasm; both

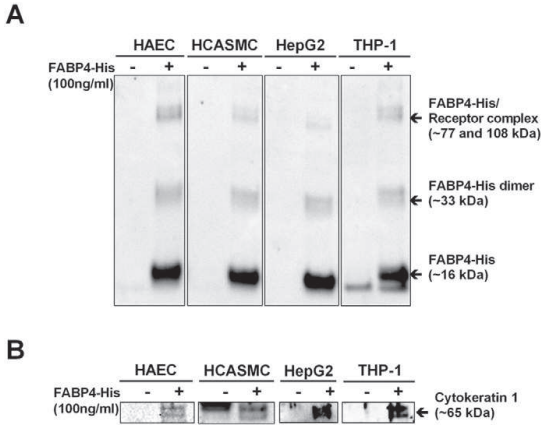

Fig. 6. Specific protein interactions of exogenous FABP4 and CK1 in HAECs, HCASMCs, HepG2 cells and THP-1 cells. FABP4 (A) and CK1 (B) western blots of plasma membrane protein lysates from HAECs, HCASMCs, HepG2 cells and THP-1 cells after incubation with or without FABP4-His (100 ng/mL) and following poly-His tag purification.

means of internalization are possible. We do know, however, that FABP4 and CK1 interact only transiently in the plasma membrane given that levels of the protein complex decreased after 5 min. In support of our results, the amount of intracellular FABP4 has been observed to increase following FABP4 incubation compared with non-treated cells [33]. It has also been shown that exogenous FABP4 can be taken up into cells [41].

We observed that administration of a FABP4 inhibitor (BMS309403) decreased complex formation between exogenous FABP4 and CK1. Therefore, fatty acids play an important role in FABP4 functionality, decreasing its interaction with plasma membrane proteins from HUVECs. BMS309403 is a biphenyl azole inhibitor designed to target FABP4. This compound competitively binds within the FABP4 fatty acid-binding pocket and inhibits the binding of FABP4 to endogenous free fatty acids [42,43]. Mechanistically, BMS309403 inhibits lipid accumulation, cholesterol efflux and inflammatory responses in macrophages, suppresses fatty acid uptake in adipocytes in a FABP4-dependent manner and stimulates glucose uptake through selective activation of the AMPK signaling pathway [43,44]. It is possible that the protection against atherosclerosis and T2DM provided by BMS309403 is due in part to a decrease in FABP4 complex formation.

This study used two proteomics strategies, both of which identified CK1 as the FABP4-binding protein in the plasma membranes of HUVECs. First, using MALDI TOF/TOF mass spectrometry, we analyzed plasma membrane protein lysates from HUVECs incubated with or without FABP4-His after cross-linking and purification with cobalt resin. The MALDI TOF/TOF profiles revealed that CK1 was the putative FABP4-binding protein. We repeated the procedure but used GeLC-MS/MS for the analysis, which also showed that CK1 was the putative receptor. CK1 was again confirmed to be the FABP4 receptor by immunoblot.

Cytokeratins are known to be part of a family of intermediate filament proteins that participate in cytoskeletal assembly [45]. CK1 is a member of the basic-neutral subfamily of cytokeratins [46]. In endothelial cells, CK1 appears to play a role as an anchor or receptor for various active molecules [45]. CK1 was also shown to colocalize with uPAR to form a multiprotein receptor complex for high-molecular-weight kininogen binding at the cell surface [47,48]. Furthermore, it was demonstrated that endothelial oxidative stress increases CK1 cell-surface protein expression and its ability the bind proteins (mannose-binding lectin) [49]. In addition, CK1 has been determined to be involved in vascular biology by regulating nitric oxide (NO) production [45], acting as a scaffolding protein for the assembly of the vasoregulatory plasma kallikrein–kinin system [40]. The kallikrein–kinin system was first recognized as a plasma and tissue proteolytic system that is responsible for the liberation of bradykinin, a vasoactive, proinflammatory mediator, resulting in NO synthesis and liberation. Tissue plasminogen activator release, superoxide formation, and prostacyclin formation are also induced by bradykinin [45]. It is known that oxidative stress and circulating FABP4 levels are increased in metabolic diseases, such as T2DM and obesity [19–25,50,51]. In our study, we hypothesized that oxidative stress could be involved in FABP4-CK1 interaction. Previous results from our group showed that exogenous FABP4 has a functional role in endothelial cells, inducing endothelial dysfunction. Exogenous FABP4 inhibits the activation of the insulin-signaling pathway, resulting in decreased eNOS activation and NO production [33]. Therefore, the FABP4-CK1 binding could decrease NO production and induce endothelial dysfunction. We also demonstrated that FABP4 inhibition decreases complex formation, likely by ameliorating endothelial dysfunction.

Furthermore, CK1 can be phosphorylated, suggesting that kininogen binding may induce intracellular signaling [45]. Exogenous FABP4 is also able to activate intracellular pathways (MAPK and Akt-dependent pathways) [33,34]; thus, it is possible that this activation also occurs through the FABP4-CK1 interaction.

We showed that this interaction did not occur in HUVECs only but that it also took place in other cell types, such as HAECs, HCASMCs, HepG2 cells and THP-1 cells. These results suggest that high levels of FABP4 in the circulation are not simply a clinical manifestation of cardiometabolic risk but are also a causative factor for cardiovascular disease. It is especially striking that exogenous FABP4 was able to form a protein complex with CK1 in HepG2 cells because these cells do not express FABP4, supporting the importance of the interaction of circulating FABP4 with peripheral tissues.

In conclusion, we demonstrated that FABP4 is not just a biomarker of metabolic diseases. Circulating FABP4 interacts with plasma membrane proteins, specifically CK1. Furthermore, FABP4 is able to cross the plasma membrane into the cytoplasm and reach the nucleus, although its role in this location is not fully known. Pharmacological inhibition of FABP4 can modulate its mechanism of action, decreasing complex formation and therefore reducing activity associated with the FABP4-CK1 interaction. Therefore, FABP4 represents a potential therapeutic target for the prevention of cardiovascular diseases that are associated with obesity and T2DM. Thus, the findings from this study and others suggest that FABP4 inhibition should be explored as a potential therapeutic strategy for treating atherosclerosis and reducing cardiovascular risk.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.09.002.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgments

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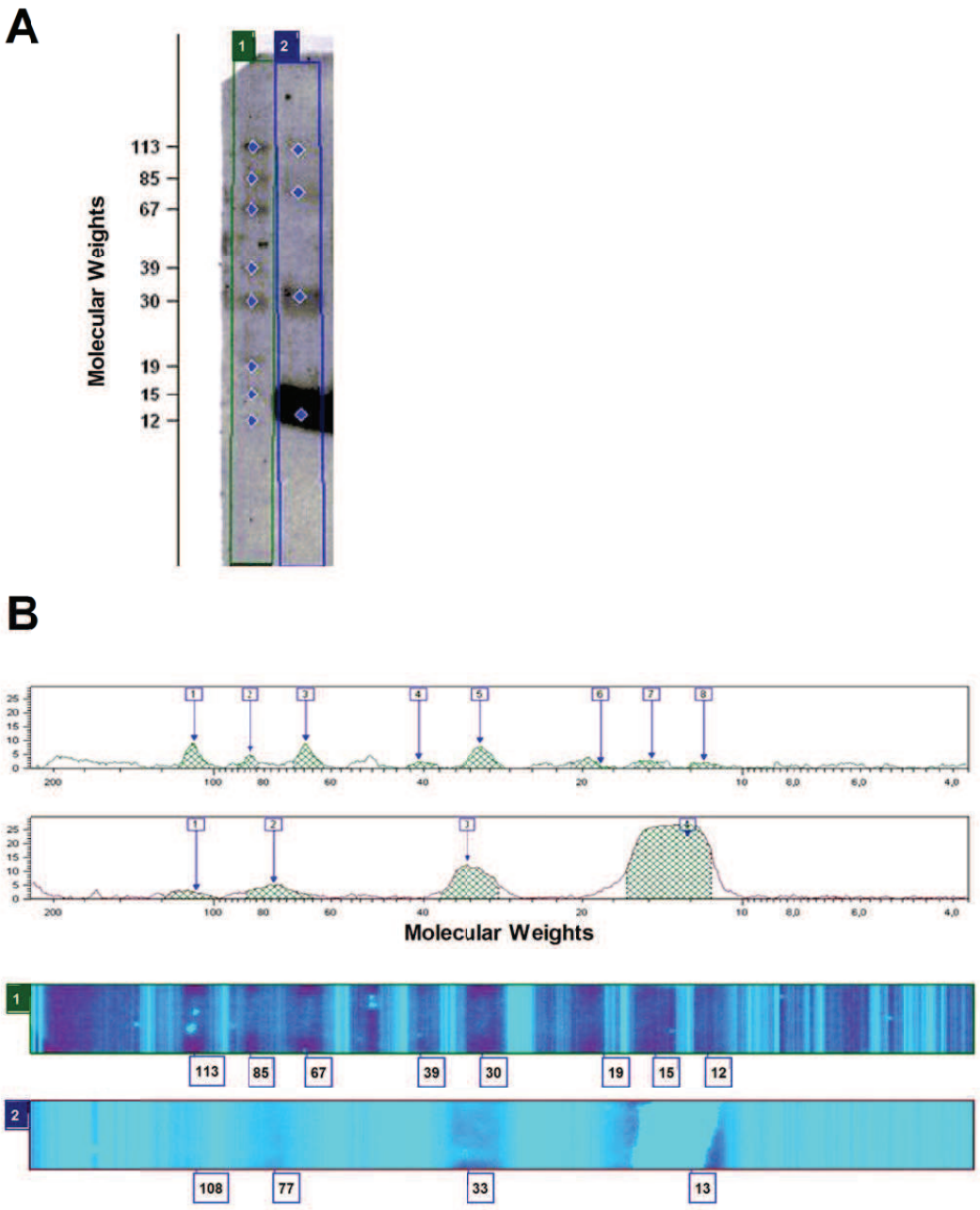
Supplementary Figure 1S



Supplementary Figure 1S. Weight band assignment of FABP4 complexes. (A) Western blot of BenchMark and plasma membrane protein lysates from HUVECs after incubation with and without FABP4-His and after poly-His tag purification, using antibodies against FABP4. (B) Weight band assignment graphics were created with ImageQuant TL v7.0 v.

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FABP4 Induces Vascular Smooth Muscle Cell Proliferation and Migration through a MAPK-Dependent Pathway

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Abstract

Purpose: The migration and proliferation of vascular smooth muscle cells play crucial roles in the development of atherosclerotic lesions. This study examined the effects of fatty acid binding protein 4 (FABP4), an adipokine that is associated with cardiovascular risk, endothelial dysfunction and proinflammatory effects, on the migration and proliferation of human coronary artery smooth muscle cells (HCASMCs).

Methods and Results: A DNA 5-bromo-2′-deoxy-uridine (BrdU) incorporation assay indicated that FABP4 significantly induced the dose-dependent proliferation of HCASMCs with a maximum stimulatory effect at 120 ng/ml (13% vs. unstimulated cells, $p<0.05$). An anti-FABP4 antibody (40 ng/ml) significantly inhibited the induced cell proliferation, demonstrating the specificity of the FABP4 proliferative effect. FABP4 significantly induced HCASMC migration in a dose-dependent manner with an initial effect at 60 ng/ml (12% vs. unstimulated cells, $p<0.05$). Time-course studies demonstrated that FABP4 significantly increased cell migration compared with unstimulated cells from 4 h (23%vs. 17%, $p<0.05$) to 12 h (74%vs. 59%, $p<0.05$). Pretreatment with LY-294002 (5 μM) and PD98059 (10 μM) blocked the FABP4-induced proliferation and migration of HCASMCs, suggesting the activation of a kinase pathway. On a molecular level, we observed an up-regulation of the MAPK pathway without activation of Akt. We found that FABP4 induced the active forms of the nuclear transcription factors c-jun and c-myc, which are regulated by MAPK cascades, and increased the expression of the downstream genes cyclin D1 and MMP2, CCL2, and fibulin 4 and 5, which are involved in cell cycle regulation and cell migration.

Conclusions: These findings indicate a direct effect of FABP4 on the migration and proliferation of HCASMCs, suggesting a role for this adipokine in vascular remodelling. Taken together, these results demonstrate that the FABP4-induced DNA synthesis and cell migration are mediated primarily through a MAPK-dependent pathway that activates the transcription factors c-jun and c-myc in HCASMCs.

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Introduction

The proliferation and directed migration of abnormal vascular smooth muscle cells (VSMCs) from the media into the intima play major roles in the pathogenesis of atherosclerotic lesions, the occurrence of restenosis after angioplasty, and the accelerated arteriopathy after cardiac transplantation[1]. Furthermore, the activation of VSMCs is a key event in the formation of the fibrous cap and the neointima. These processes are triggered by multiple cytokines and growth factors, such as tumour necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), and transforming growth factor-β (TGF-β), among others, and mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt are the two major signalling pathways linked to migration and proliferation[2,3]. Understanding the potential mechanisms governing VSMC migration and proliferation may provide new perspectives in the effort to inhibit this inflammatory process.

The adipose fatty acid-binding protein (FABP), also known as FABP4 and aP2, is one of the most well-characterised intracellular lipid transport proteins[4]. It belongs to a

superfamily of low-molecular-weight intracellular lipid-binding proteins and plays a central regulatory role in energy metabolism and inflammation[5-7]. FABP4 is highly expressed in mature adipocytes and accounts for approximately 6 % of the soluble protein in the adipocyte. FABP4 is also found in circulating blood plasma. In the last several years, much effort has been focused on uncovering the role of FABP4. However, neither the secretory pathways nor the functions of circulating FABP4 are known. We and other authors have shown that FABP4 levels are increased in obesity, metabolic syndrome (MS), type 2 diabetes (T2D), and familial combined hyperlipidaemia or lipodystrophy syndromes and that these increased levels are also closely correlated with adverse lipid profiles and insulin resistance[8-14]. In these and other studies, serum FABP4 predicted the development of MS and atherosclerosis[15-17]. Moreover, increased plasma levels of FABP4 in non-elderly men were independently associated with the presence of coronary artery disease[18]. In addition, FABP4 is found in human atherosclerotic plaques, and its presence is associated with high-risk atherosclerotic plaques such as unstable, inflammatory and vulnerable plaques[19-22].

FABP4 has been implicated in several critical cellular processes, such as the uptake and intracellular storage of fatty acids and the regulation of gene expression, cell proliferation, and differentiation[23]. In addition to being expressed in adipocytes and macrophages, the constitutive or induced expression of FABP4 has been found in coronary endothelial cells, trophoblasts, muscle cells and epithelial cells, suggesting additional biological roles[24,25]. A recent study demonstrated that FABP4 decreased the contractility of myocardial muscle cells, which suggests that the release of FABP4 into the bloodstream could have a direct effect on some peripheral cells and tissues[26]. In addition, we recently demonstrated that high levels of plasma FABP4, as well as other inflammation mediators, were associated with endothelial dysfunction as assessed by peripheral artery tonometry[27,28], and in an in vitro study, we previously demonstrated that recombinant FABP4 causes endothelial dysfunction by impairing the insulin-signalling pathway and NO production[29]. Furthermore, the elevated expression of intracellular FABP4 in endothelial cells was found to contribute to the dysfunction of these cells by reducing eNOS[30]. The knockdown of FABP4 in endothelial cells significantly reduces the proliferation of these cells both under baseline conditions and in response to VEGF and bFGF[24]. Although all of these data suggest a role for FABP4 in vascular dysfunction, there are no data regarding the effect of FABP4 on human coronary artery smooth muscle cell (HCASMC) activation.

These data, along with our own observations illustrating the influence of circulating FABP4 on vascular function, support testing the hypothesis that the high levels of circulating FABP4 in altered metabolic conditions could modify the normal function of VSMCs and cause proliferation and migration. The aims of the current study were to evaluate the direct influence of FABP4 on HCASMC proliferation and migration and to analyse the intracellular signalling pathways involved.

Material and Methods

Cell culture and reagents

Primary HCASMCs were obtained from Cascade BiologicsTM (Invitrogen Life Technologies, Paisley, UK). After thawing, the cells were seeded into 75 cm^2 flasks and cultured according to the supplier's recommendations in Medium 231. Medium 231 was supplemented with smooth muscle growth supplement (SMGS) and 1% gentamicin/amphotericin solution (Invitrogen Life Technologies, Paisley, UK). The supplemented medium contained foetal bovine serum (4.9 % v/v final concentration), human basic fibroblast growth factor (2 ng/ml), human epidermal growth factor (0.5 ng/ml), heparin (5 ng/ml), insulin (5 µg/ml) and BSA (0.2 µg/ml). The cells were placed in a humidified incubator at 37°C and 5% CO_2 until there were enough cells available for the experiments. In the current study, the HCASMCs were used at passage 6. Before the initiation of the assays, HCASMCs that were in exponential growth were switched into Medium 231 supplemented with 0.1% FBS in the absence of growth factors for 24 h to achieve cell quiescence.

The cytotoxicity assay was performed by analysing LDH release into the medium using the Cytotoxicity Detection Kit (Roche Diagnostics, Basel, Switzerland). TNFα was purchased from Calbiochem (Merck, Darmstadt, Germany). To study FABP4 activation pathways in HCASMCs, the PI3K inhibitor LY294002 and the MAPK inhibitor PD98059 (Calbiochem-Merck, Darmstadt, Germany) and the c-Jun inhibitor SP600125 and the c-Myc inhibitor 10074-G5 (Sigma-Aldrich, Madrid, Spain) were used. Human recombinant FABP4 and the anti-FABP4 antibody were obtained from BioVendor (Brno, Czech Republic). The anti-Akt, anti-pAkt (Ser^{473}), anti-p44/p42 MAPK (Erk1/2), anti-phospho-p44/p42 MAPK (Erk1/2) Thr^{202}/Tyr^{204}, anti-NF-kB p65 and anti-cyclin D1 antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies specific for the active forms of the following transcription factors were used: phospho-ATF2 (Thr^{71}), phospho-c-jun (Ser^{73}), c-myc and STAT1α were purchased from Active Motif (La Hulpe, Belgium). Anti-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and IgG-HRP was purchased from Dako (Glostrup, Denmark).

Cell proliferation studies

Cell proliferation was analysed by measuring DNA synthesis with a colorimetric bromodeoxyuridine (BrdU) enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions. Briefly, $1x10^4$ cells were seeded into a 96-well microplate and cultured with or without FABP4 (30-120 ng/ml), TNF-α (10 ng/ml), anti-FABP4 antibody (40 ng/ml) and/or LY294002 (5 µM), PD98059 (10 µM), 10074-G5 (10 µM) and SP600125 (10 µM) for 24 h. The cells were then labelled with BrdU labelling reagent for 10 h. After fixation, the cells were incubated with anti-BrdU antibody for 90 min. After washing, 100 µl of substrate (tetramethylbenzidine) was added to each well, and the plates were incubated at room temperature for 30 min. The absorbance at 450 nm was measured with an ELISA reader (Synergy H4, Biotek, USA).

In vitro wound-healing assay

Cell migration was analysed with the in vitro scratch assay[31]. The cells were cultured in 12-well plates, and after the induction of quiescence, a single scratch wound was created in the centre of the cell monolayer by the gentle removal of the attached cells with a sterile plastic pipette tip. The cells were incubated with FABP4 (30-240 ng/ml) and/or LY294002 (5 μM), PD98059 (10 μM), 10074-G5 (10 μM) and SP600125 (10 μM) for 24 h in serum-reduced Medium 231 (containing 0,1 % foetal calf serum in the absence of growth factors). Images of the cells migrating into the wound were taken at 0 h and then every 2 h until the scratch wound was closed at 24 h; the images were compared to quantify the migration rate of the cells. The closure of the wound was considered to represent 100% migration. The cell images were captured using a microscope (Olympus IX71, Spain) and analysed using imaging software (Xcell). To investigate the effect of FABP4 on fibulin 4, fibulin 5, MMP2, and CCL2 expression, migrating HCASMC were incubated with 120 ng/ml of FABP4 in the presence or absence of c-myc (10074G5) and c-jun (SP600125) inhibitors for 24 h. Briefly, the cells were plated in 8.8-cm^2 culture dishes. After the induction of quiescence, a cross scratch wound was created by the gentle removal of the attached cells with a sterile plastic Pasteur pipette. The cells were incubated with FABP4 with or without inhibitors, and 24 h later, the migrating cells were scraped from the plate under microscopic observation.

Total cellular and nuclear extracts

To obtain total cellular extracts, HCASMCs were cultured in 10 cm culture dishes until the cells reached 90% confluence. After the induction of quiescence, the cells were incubated with FABP4 (120 ng/ml) or TNF (30 ng/ml) for 5, 15 or 30 min. At different time points, the cells were rinsed with ice-cold PBS and lysed in lysis buffer, which was composed of 50 mM Tris–HCl, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate and phosphatase inhibitors (Roche Diagnostics, Basel, Switzerland). To investigate the effect of FABP4 on cyclin D1 expression, proliferating HCASMC were incubated with 120 ng/ml FABP4 in the presence or absence of c-myc (10074G5) or c-jun (SP600125) inhibitors for 6 h. The cells were then lysed, and total extracts were analysed for cyclin D1 expression by western blotting. The cells were then stored at −80°C until they were processed. The total protein concentration was measured using a Bradford assay kit (BioRad, USA), and the immunoblot analysis was then performed. For the nuclear extracts, HCASMCs were cultured in 10 cm culture dishes until the cells reached 90% confluence. After the induction of quiescence, the cells were incubated with FABP4 (120 ng/ml) or TNF (30 ng/ml) for 15 min or 2 or 4 h. Nuclear protein extracts were prepared essentially as described below. The HCASMCs were homogenised in ice-cold hypotonic buffer (10 mM HEPES, 0.1 mM EDTA, 0.1 mM EGTA, 10 mM KCL, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT and phosphatase inhibitors) and centrifuged at 16,000xg for 10 min at 4°C. The homogenate was layered onto extraction buffer (20 mM HEPES, 25% glycerol, 0.42 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and phosphatase inhibitors) and centrifuged at 16,000×g for 60 min at 4°C. After centrifugation, the supernatant (nuclear extract) was collected, the protein concentration was measured with a Bradford assay kit (Bio-Rad, USA) and the immunoblot analysis was performed. The extracts were used immediately or stored at -80°C for later use.

Immunoblot analysis

Electrophoresis and immunoblot analysis were performed using the NuPAGE protein analysis system (Invitrogen Life Technologies, UK). The membranes were blocked with a 2% ECL Advance Blocking Reagent (Amersham Biosciences, USA) and incubated with anti-FABP4, anti-cyclin D1, anti-actin, anti-Akt, anti-phospho-Akt (Ser^{473}), anti-ERK1/2 and anti-phospho-p44/p42 MAPK (Erk1/2) Thr^{202}/Tyr^{204} antibodies, as well as anti-phospho-ATF2 (Thr^{71}), anti-phospho-c-jun (Ser^{73}), anti-c-myc, anti-STAT1α and anti-NF-kB p65 antibodies. The antigen-antibody complexes were detected by incubating the membrane with an HRP-conjugated anti-IgG antibody. The bands were visualised using ECL reagents (Amersham Pharmacia, USA) with the VersaDoc image system and quantified with the Quantity One analysis software, version 4.6.2 (Bio Rad, USA). The relative levels of the phosphorylated forms of Akt and ERK1/2 were quantified after normalisation to the total protein levels, and the levels of the activated forms of the transcription factors were expressed relative to the levels of the respective factors in the non-stimulated cells at each time point; all of the values were expressed in arbitrary units (AU).

Quantitative real-time RT-PCR

Total RNA was isolated from the cells using the ABI PRISM 6100 Nucleic Acid PrepStation (Applied Biosystems, CA, USA). The absorbance at 260 nm was used to measure the RNA concentration, and an absorbance ratio of 260/280 nm was used to analyse the quality of the RNA. Total RNA (0.5 μg) was reverse transcribed to cDNA using random hexamers and SuperScript II (Invitrogen Life Technologies, UK) following the manufacturer's protocol. TaqMan primers and probes for fibulin 4, fibulin 5, MMP2 and CCL2 were obtained from validated and pre-designed Assays-on-Demand products (Applied Biosystems, CA, USA) and were used in real-time PCR amplifications. The mRNA expression for each gene and sample was calculated using the recommended 2-ΔΔCt method. The control group (untreated cells) was defined as the calibrator in this experiment. GAPDH was used as a housekeeping gene to normalise the results of the gene of interest.

Statistical analysis

The results were represented as the means ± SEM of at least 3 separate experiments. Differences between the means were determined using a t test or a one-way analysis of variance (ANOVA), which was followed by a Dunnett's post-hoc test for multiple comparisons. The differences were considered to be significant at $p<0.05$. The GraphPad Prism 5.0 software, GraphPad Software Inc. was used for the statistical analyses.

Results

Effect of FABP4 on human coronary artery smooth muscle cell (HCASMC) proliferation

To assess the effect of FABP4 on HCASMC proliferation, we performed dose-response experiments using BrdU uptake as a marker for DNA synthesis. The BrdU incorporation indicated that, when HCASMCs were stimulated with increasing concentrations of FABP4 (30-120 ng/ml), a significant dose-response effect on cell proliferation at 24 h was observed (Figure 1). FABP4 had no effect on HCASMC proliferation at 30 ng/ml, while a significant increase in proliferation was observed at 60 ng/ml and 120 ng/ml (12% and 13% vs. unstimulated cells, respectively, $p<0.05$). TNF-α at 10 ng/ml, which was chosen as the positive control because of the well-known mitogenic effect of TNF-α on SMCs, resulted in an increase of 17% with respect to the unstimulated cells ($p<0.05$). Cell proliferation was also measured in the presence of an anti-FABP4 antibody (40 ng/ml) to analyse the specific effect of FABP4. The presence of the anti-FABP4 antibody completely inhibited the proliferation induced by FABP4 at concentrations of 60 ng/ml and 120 ng/ml ($p<0.05$) (Figure 1A). Because PI3K and MAPK pathways are the primary regulators of cell proliferation, we investigated the effects of LY294002, a selective inhibitor of PI3K and of PD98059, a selective inhibitor of MAPK, by BrdU uptake measurement. As shown in Figure 1B, LY294002 (5 μM) and PD98059 (10 μM) significantly blocked FABP4-induced HCASMC proliferation ($p<0.05$), suggesting that both pathways areinvolved in FABP4-induced cell proliferation.

Effect of FABP4 on human coronary artery smooth muscle cell (HCASMC) migration

To address the effect of FAPB4 on HCASMC migration, a wound-healing assay was performed. We analysed the cell migration every 2 h for 24 h. As shown in Figure 2A, FABP4-treated (60 ng/ml) HCASMCs migrated earlier than the untreated cells and almost completely closed the denuded area after 12 h of treatment (Figure 2A). As shown in Figure 2B, dose-response studies (30-240 ng/ml) revealed that FABP4 significantly increased cell migration at 60 ng/ml, 120 ng/ml and 240 ng/ml with respect to the untreated cells at 6 h (12%, $p<0.05$; 34%, $p<0.05$; and 51%, $p<0.05$, respectively). As shown in Figure 2C, the time-course studies (0 h-24 h) revealed that FABP4 (60 ng/ml) significantly increased the migration of HCASMCs compared with the untreated cells, with a 6% increase within 4 h of treatment ($p<0.05$). This significant increase in cell migration was observed at each time point, with a maximum effect (an increase of 15%) after 12 h of treatment ($p<0.05$). Both treated and untreated cells reached 100% migration at the 24 h time point (Figure 2C).

To investigate the roles of the PI3K and MAPK pathways in cell migration, HCASMCs were incubated with FABP4 (120 ng/ml) and LY294002 (5 μM) or PD98059 (10 μM) for 6 h, and their migration rate was measured. The results showed that the addition of MAPK inhibitor completely blocked FABP4-induced HCASMC migration at 6 h ($p<0.05$, Figure 2D). Together with the partial inhibition of migration observed with the PI3K inhibitor (LY294002), these results indicate that the MAPK pathway is the main pathway responsible for the FABP4-mediated increase in cell migration.

No cytotoxic effect, measured as LDH leakage, was observed in any of the experiments performed.

Effect of FABP4 on ERK1/2 and AKT activation in human coronary artery smooth muscle cells (HCASMCs)

As reported in previously published literature, VSMC proliferation and migration are mediated via the ERK and Akt pathways[2,3]. Therefore, the effects of FABP4 on the activation of ERK1/2 and Akt were explored. Cells were incubated with FABP4 (120 ng/ml) for 5, 15 or 30 min, and the total cell extracts were analysed. The results indicated that FABP4 rapidly and significantly activated ERK1/2 phosphorylation in as little as 5 min (4.8-fold increase, $p<0.05$); the phosphorylation returned to basal levels after 30 min (Figure 3A). As shown in Figure 3B, FABP4 had no effect on AKT activation. TNF-α (10 ng/ml), which was used as a control, activated ERK1/2 and AKT phosphorylation at 5 and 30 min, respectively (5.2 and 1.35-fold increases, respectively) (Figure 3C).

We next analysed whether extracellular FABP4 affects the cellular pool of FABP4. Figure S1 shows that the incubation of HCASMCs with 120 ng/ml FABP4 for 5, 15 or 30 min increased the cellular pool of FABP4 2-fold compared to non-treated cells ($p<0.05$).

Effect of FABP4 on transcription factors activated by the MAPK signal transduction pathway in human coronary artery smooth muscle cells (HCASMCs)

Next, we investigated the effect of FABP4 on the transcription factors activated by the MAPK signal transduction pathway. HCASMCs were incubated with FABP4 (120 ng/ml) for 15 min, 2 h or 4 h, and the nuclear cell extracts were isolated. We analysed the effect of FABP4 on the active forms of the following transcription factors: phospho-ATF2 (Thr^{71}), phospho-c-jun (Ser^{73}), c-myc, STAT1α and NF-kB p65. As shown in Figure 4, the nuclear extracts of the cells incubated with FABP4 (120 ng/ml) for 2 h tended to yield increases in the active forms of all of the transcription factors analysed, with increases in the active forms of c-jun (1.43±0.01, $p<0.05$) and c-myc (1.43±0.06, $p<0.05$) reaching statistical significance. To confirm the involvement of the transcription factors c-jun and c-myc in FABP4-induced HCASMC proliferation and migration, HCASMCs were incubated with FABP4 (120 ng/ml) and SP600125 (10 μM) or 10074-G5 (10 μM) for 24 h and BrdU incorporation was measured. We found that SP600125 and 10074-G5 significantly attenuated FABP4-induced HCASMC proliferation ($p<0.05$) (Figure 5, left axis). In another experiment, HCASMCs were incubated with FABP4 (120 ng/ml) and SP600125 (10 μM) or 10074-G5 (10 μM) for 6 h, and their migration rate was measured. Both inhibitors blocked the stimulatory effect of FABP4 on cell migration ($p<0.05$) (Figure 5, right axis). We conclude that the activation of both c-jun and c-myc is involved in FABP4-induced cell proliferation and migration.

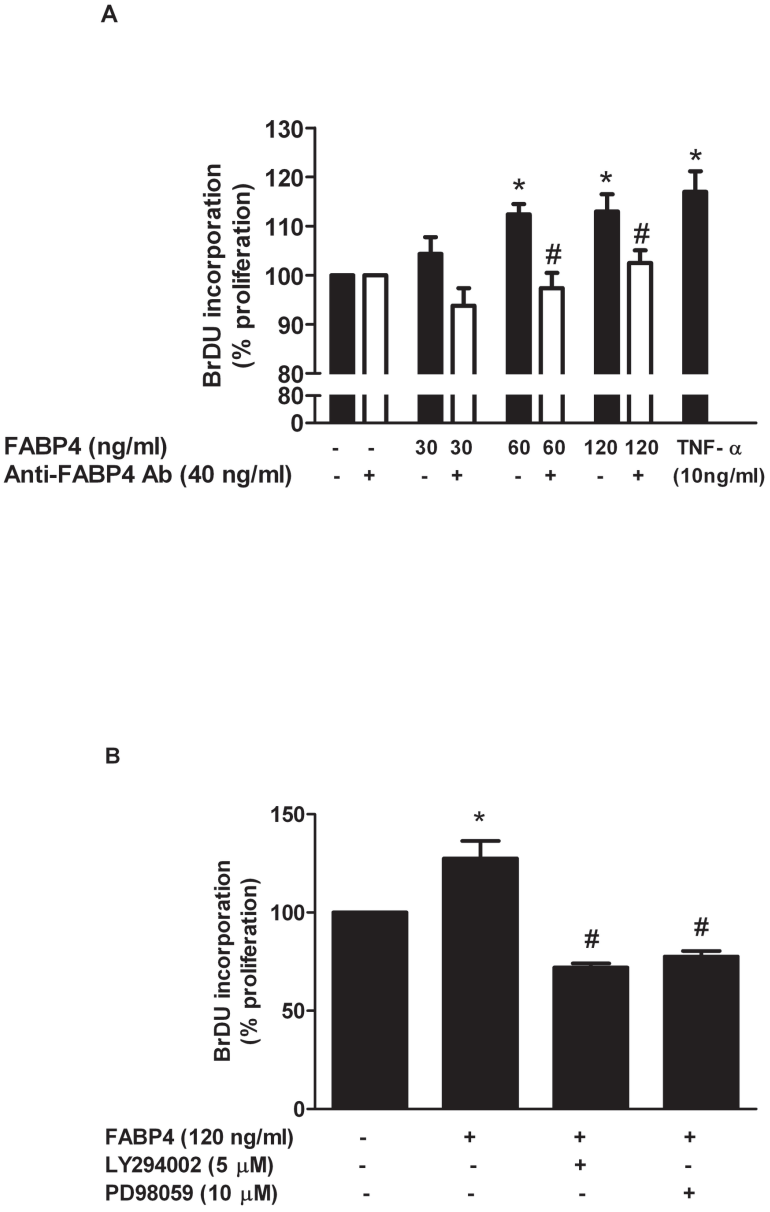

Figure 1. Effect of FABP4 on HCSMCs proliferation as assessed by BrdU incorporation. (A) Effect of FABP4 (30-120 ng/ml) with and without anti-FABP4 antibody (40 ng/ml) on the proliferation HCASMCs at 24 h. TNF-α (10 ng/ml) was used as a positive control. (B) Effect of LY294002 (5 μM) and PD98059 (10 μM) on FABP4 (120 ng/ml)-induced HCASMCs proliferation at 24 h. The results are expressed as the mean ± SEM of three experiments run in quadruplicate. *p<0.05 vs unstimulated cells; #p<0.05 vs FABP4-stimulated cells.

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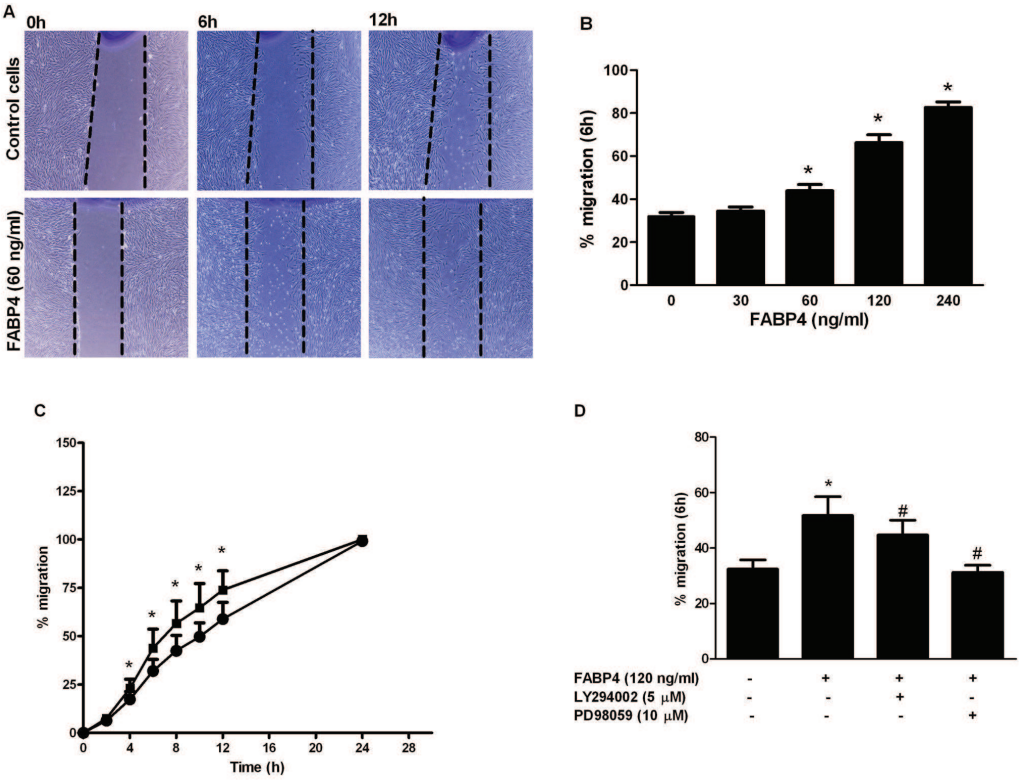

Figure 2. Effect of FABP4 on HCASMC migration. (A) An *in vitro* wound-healing assay indicates that FABP4 at 60 ng/ml induced HCASMC migration. Confluent cells were scratch wounded and allowed to migrate for 12 h. Representative images of the cells after 0 h, 6 h and 12 h of migration are shown. Photomicrographs of the cell images were obtained at 100x magnification in a phase-contrast microscope. (B) The effects of FABP4 (30-240 ng/ml) on HCASMC migration after 6 h of treatment. The results are shown as the percentage of increase in relation to the cells treated with medium alone (control). (C) A time-course (0-24 h) of the effect of 60 ng/ml FABP4 on HCASMC migration. (D) The effect of LY294002 (5 μM) and PD98059 (10 μM) on FABP4-induced HCASMCs at 6 h. *p<0.05 vs unstimulated cells; #p<0.05 vs FABP4-stimulated cells. The results are shown as a percentage of the migration rate. The results are expressed as the mean ± SEM of three experiments run in sextuplicate. *p<0.05 vs. unstimulated cells (control).

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Effect of FABP4 on the expression of cyclin D1, MMP2, CCL2, and fibulin 4 and 5

Cyclin D1 and MMP2, CCL2, and fibulin 4 and 5, which are involved in cell cycle regulation and cell migration, respectively, are downstream genes of the c-Fos and c-myc transcription factors. To investigate the effect of FABP4 on cyclin D1 expression, proliferating HCASMC were incubated with 120 ng/ml FABP4 in the presence or absence of the inhibitors 10074G5 (10 μM) or SP600125 (10 μM) for 6 h. The results showed that FABP4 increases cyclin D1 expression 1.9-fold (Figure 6A). The addition of the c-jun inhibitor SP600125 had no effect on FABP4-induced cyclin D1 expression, while the addition of the c-myc inhibitor 10074G5 caused the expression of cyclin D1 to return to basal levels (Figure 6A).

To investigate the effect of FABP4 on fibulin 4, fibulin 5, MMP2, and CCL2 expression, migrating HCASMC were incubated with 120 ng/ml FABP4 in the presence or absence of 10074G5 (10 μM) and SP600125 (10 μM) for 24 h. The results showed that FABP4 significantly increased the mRNA levels of all genes studied ($p<0.05$); the greatest effect was observed for MMP2 mRNA, the level of which increased 3-fold (3.1±0.2, $p<0.05$)(Figure 6B). The addition of 10074G5 (10 μM) and SP600125 (10 μM) reversed this effect for all the genes studied ($p<0.05$) (Figure 6B).

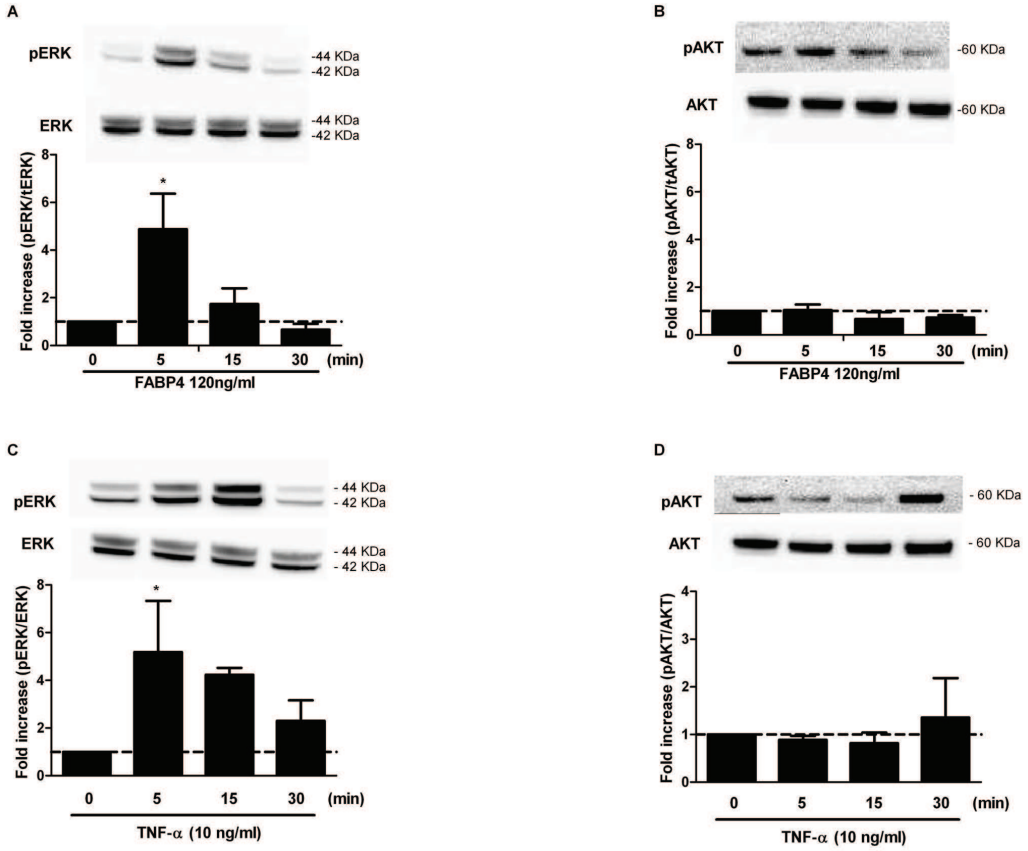

Figure 3. Effect of FABP4 (120 ng/ml) on ERK1/2 and AKT activation in HCASMCs. Effect of FABP4 (120 ng/ml) on ERK1/2 (A) and AKT (B) activation in HCASMCs. TNF-α (10 ng/ml) was used as the positive control (C, D). Representative western blots and relative densitometric analyses are shown. The results are expressed as the mean ± SEM of three separate experiments. *p<0.05 vs. unstimulated cells (control).

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Discussion

Increases in VSMC proliferation and migration are key events in the pathogenesis of atherosclerosis, as well as intimal hyperplasia after vascular injury[32]. FABP4 exhibits biological activity in various cell types to promote a proinflammatory state and vascular dysfunction[7]; however, nothing was previously known about the direct influence of FABP4 on HCASMC activation and the intracellular signalling pathways involved. In the present study, we demonstrated that FABP4 directly promotes the in vitro proliferation and migration of HCASMCs through the activation of the ERK1/2 MAPK signalling pathway. We also demonstrated that FABP4 activates the nuclear transcription factors c-myc and c-jun and that it increases the expression of their downstream genes cyclin D1 and MMP2, CCL2, and fibulin 4 and 5, which are involved in cell cycle regulation and cell migration, respectively.

It is well known that FABP4 is an adipocyte- and macrophage-produced protein that promotes insulin resistance, hypertriacylglycerolaemia and atherosclerosis and is also a circulating protein, the levels of which are independently and positively associated with metabolic syndrome and vascular disease. Data from animal studies also support the pathogenic role of FABP4 in cardiovascular disease[33,34]. However, little is known concerning the role of FABP4 as a secreted adipokine, including the target tissues, the actions of the protein and the underlying mechanisms. FABP4 might be released into the circulation at least in part by adipocyte-

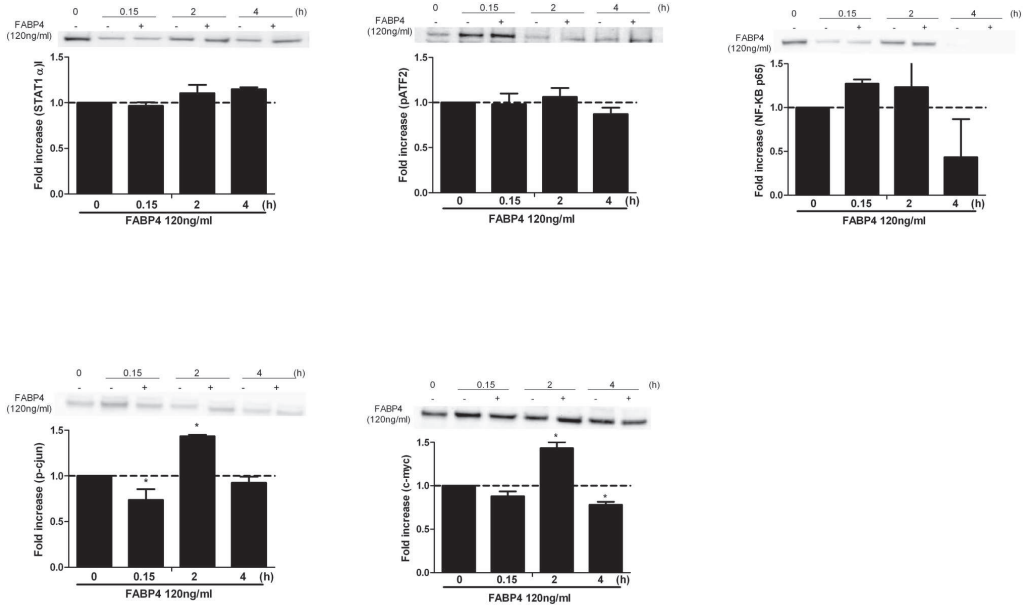

Figure 4. Effect of FABP4 (120 ng/ml) on STAT1α, ATF2, NF-Kb p65, c-jun and c-myc activation in HCASMCs. Representative western blots and relative densitometric analyses are shown. The results are expressed as the mean ± SEM of three separate experiments. *p<0.05 vs. unstimulated cells (control).

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derived microvesicles or from the lysis of large adipocytes[8]. Our results support a direct effect of extracellular FABP4 on peripheral tissues, specifically on HCASMCs. There are few studies indicating a direct effect of FABP4 on cells. Lamounnier-Zepter et al. demonstrated that recombinant FABP4 reduces the contractile capacity of cardiomyocytes, illustrating a cardiodepressant activity of the factor[26]. In addition, we have previously shown that exogenous FABP4 impairs endothelial function by inhibiting the activation of the insulin signalling pathway, resulting in decreased eNOS activation and NO production[29].

Our present results demonstrate that recombinant FABP4 induces dose-dependent HCASMC proliferation. We found that this effect is specific because the addition of an anti-FABP4 antibody inhibited the cellular proliferation. In addition to cell proliferation, we found that FABP4 also increases cell migration in a dose- and time-dependent manner. Our findings are in good agreement with a recently published study showing that recombinant and secretory FABP4 cause an enhancement of growth and migration in human aortic smooth muscle cells through a ROS-mediated mechanisms[35]. Using another cell type, Elmasri et al. revealed a novel pro-angiogenic role for endothelial cell FABP4, which promoted the migration and invasion of HUVECs[36]. The authors showed that FABP4 deficiency alone was sufficient to induce apoptosis in HUVECs. Furthermore, the knockdown of FABP4 dramatically reduces the proliferation of endothelial cells both under baseline conditions and in response to VEGF[24]. The induction of cultured HCASMC migration and proliferation by FABP4, as demonstrated in the current study, suggests a potential role for this adipokine in promoting vascular pathology.

We studied different mechanisms for FAPB4-induced HCASMC proliferation and migration. The involvement of the ERK1/2 MAPK cascade and the PI3K/AKT signalling pathway in SMC proliferation and migration has previously been demonstrated[2,3]. The c-Raf/MEK/ERK pathway is essential for cell proliferation and migration[37], and our results indicate that FABP4 increases the phosphorylation of ERK1/2, which suggests that FABP4 utilises the ERK pathway to induce smooth vascular cell proliferation and migration. We found that although FABP4 had no effect on Akt activation, an inhibitor of PI3K (LY294002) blocked the effects of FABP4 on HCASMC proliferation and migration, which suggests the activation of an additional kinase pathway. Crosstalk between the Ras/c-Raf/MEK/ERK pathway and other signalling pathways appears to occur. For example, RAS can activate the PI3K/AKT pathway, in addition to having other shared inputs, and there appears to be some compensation for the loss of signalling activity when one pathway or the other is inhibited. Moreover, the selective inhibition of MAPK kinase by PD98059

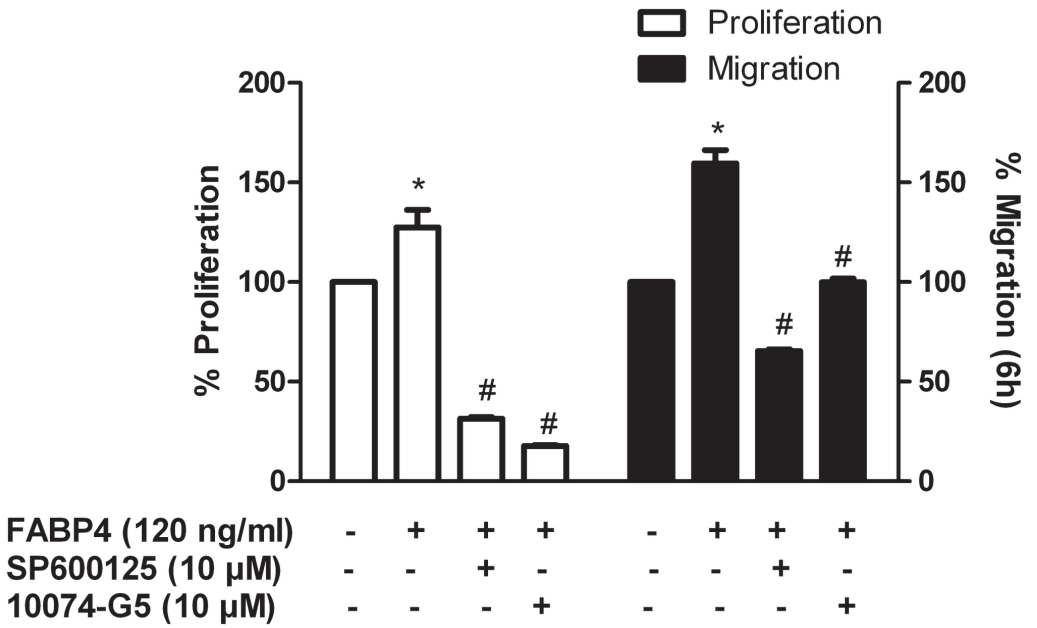

Figure 5. Effects of the c-jun inhibitor (SP600125) and the c-myc inhibitor (10074-G5) on FABP4-induced HCASMC proliferation and migration. Effect of SP600125 (10 μM) and 10074-G5 (10 μM) on FABP4 (120 ng/ml)-induced HCASMC proliferation at 24 h and on cell migration at 6 h. The results are expressed as mean±SEM of three separate experiments run at least in triplicate. *p<0.05 vs unstimulated cells; #p<0.05 vs FABP4-stimulated cells.

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significantly reduced FABP4-induced HCASMC proliferation and migration. Taken together, these data clearly demonstrate that FABP4-induced HCASMC proliferation and migration are dependent on ERK1/2 and PI3K activation without the direct involvement of Akt activation. The translocation of phosphorylated ERK to the nucleus can activate transcription factors such as c-myc and c-jun, and this type of activation was demonstrated in the present study. Thus, our results suggest that the signals initiated by FABP4 activate the c-Raf/MEK/ERK pathway, resulting in the induction of the c-myc and c-jun transcription factors. Although the observed increases in the levels of these transcription factors were not striking, the addition of specific inhibitors of c-jun (SP600123) and c-myc (10074G5) significantly attenuated FABP4-induced HCASMC proliferation and migration. It has been widely observed that modest changes on transcription factor levels can have a great impact on the concentrations regulated proteins. Cyclin D1 and MMP2, CCL2, and fibulin 4 and 5 are downstream genes of c-Fos and c-Myc transcription factors and are involved in cell cycle regulation and cell migration, respectively. In this study, we found that the treatment of HCASMCs with FABP4 increased cyclin D1 expression and cell proliferation through c-myc, as demonstrated by the addition of inhibitors of c-jun and c-myc. This effect was more striking when we evaluated the expression of genes involved in cell migration (MMP2, CCL2, fibulin 4 and 5). Thus, it is conceivable that increases in MAPK signalling by extracellular FABP4 lead to increased levels of cell migration-related proteins rather than increased levels of proteins related to cell proliferation.

It is not known whether extracellular FABP4 is internalised into the cell or whether it acts by an intracellular mechanism. We observed that the cellular pool of FABP4 increased after FABP4 incubation, which suggests that FABP4 can bind to the cell membrane of HCASMCs and/or be internalised into the cells.

The concentrations of FABP4 used in the present study are higher than the levels observed in vivo. The FABP4 doses used in our study were those that we found to have a biological effect on the insulin-dependent nitric oxide pathway in HUVEC[29]. Notably, the concentrations of recombinant FABP4 used in other in vitro studies were ~15 to 100 times higher than the concentrations used in our study. Lamounier-Zepter et al.[26] used ~1500 ng/ml of FABP4 to show an effect in cardiomyocytes, and Lu et al.[35] used 2500 to 10000 ng/ml of FABP4 to show an effect in aortic SMCs. Thus, it seems that higher concentrations of FABP4 than those observed in vivo are required to produce significant effects in vitro. In addition, we cannot rule out that the concentration of a biomarker, in this

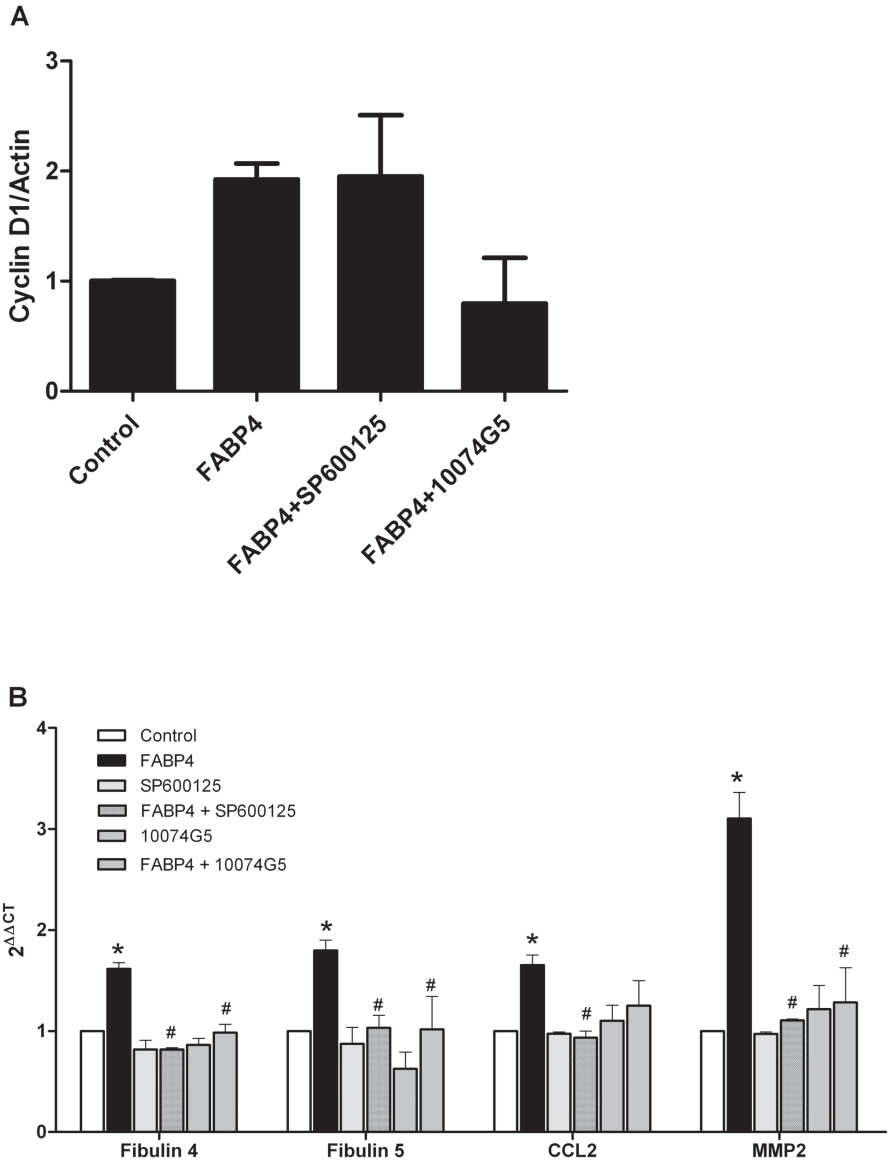

Figure 6. Effect of FABP4 on target genes regulated by c-fos and/or c-myc. (A) Effect of FABP4 on cyclin D1 expression. Proliferating HCASMC were incubated with 120 ng/ml FABP4 in the presence or absence of c-myc (10074G5) and c-jun (SP600125) inhibitors for 6 h. Western blotting was performed to evaluate the expression of cyclin D1 in the cells, with actin as an internal control. (B) Migrating HCASMC were incubated with 120 ng/ml FABP4 in the presence or absence of c-myc (10074G5) and c-jun (SP600125) inhibitors for 24 h. Total RNA was extracted from the cells, and the expression of fibulin 4, fibulin 5, MMP2, and CCL2 was measured by quantitative RT-PCR using TaqMan primers and the 2ΔΔCt method. *p<0.05 vs unstimulated cells; #p<0.05 vs FABP4-stimulated cells.

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case FABP4, found in the interstitial tissue was higher than the concentration in plasma.

In conclusion, our results demonstrate that exogenous FABP4 induces VSMC migration and proliferation *in vitro*, mainly via ERK activation. Our findings suggest that high levels of FABP4 in the circulation are not simply a clinical manifestation of cardiometabolic risk but are also a causative factor in vascular pathology. Furthermore, the data from our group and other groups suggest that pharmacological inhibition of FABP4 should be explored as a potential therapeutic strategy for treating atherosclerosis and reducing cardiovascular risk.

Supporting Information

Figure S1. Presence of FABP4 in total cell lysates. HCASMCs were treated with or without FABP4 (120 ng/ml) for the indicated times. Representative blots are shown. The data represent the mean ± SEM values obtained in three independent experiments. *P<0.05 vs. without FABP4.
(TIF)

Author Contributions

Conceived and designed the experiments: JG NP LM JCV. Performed the experiments: JG RR PS JCV. Analyzed the data: JG RR JCV. Wrote the manuscript: JG LM JCV.

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5. Summary results

UNIVERSITAT ROVIRA I VIRGILI
FABP4: INTERACTIONS WITH ENDOTHELIAL CELL PLASMA MEMBRANE AND EFFECTS ON VASCULAR SMOOTH MUSCLE CELLS.
Paula Saavedra García
Dipòsit Legal: T 238-2016

- Exogenous FABP4 is found in plasma membranes of HUVECs and interacts specifically with plasma membrane proteins.
- CK1 is a FABP4-binding protein on endothelial cell membranes.
- Fatty acids are required for the formation of FABP4 protein complexes.
- Exogenous FABP4 is internalized into HUVECs, passing through the cell membrane into the cytoplasm after 30 min and into the nucleus after 2 h.
- Exogenous FABP4 also forms protein complexes with CK1 in HAECs, HCASMCs, HepG2 and THP-1 cells.
- Exogenous FABP4 promotes migration and proliferation in HCASMCs through ERK1/2 and PI3K activation inducing the active forms of the nuclear transcription factors regulated by MAPK cascades (c-jun and c-myc) and increasing the expression of the downstream genes involved in cell cycle regulation and cell migration (cyclin D1 and MMP2, CCL2 and fibulins 4 and 5).
- Intracellular FABP4 increases after FABP4-incubations in HCASMCs.

6. Discussion

UNIVERSITAT ROVIRA I VIRGILI
FABP4: INTERACTIONS WITH ENDOTHELIAL CELL PLASMA MEMBRANE AND EFFECTS ON VASCULAR SMOOTH MUSCLE CELLS.
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6.1. Exogenous FABP4 interacts specifically with CK1 on the endothelial plasma membrane

Previous studies have postulated that FABP4 is a plasmatic biomarker of MS, T2DM and atherosclerotic disease[145, 149-151]. In this study, interactions between exogenous FABP4 and the plasma membranes of HUVECs were shown, supporting the hypothesis that circulating FABP4 is not simply a biomarker but that it may have a causal effect by interacting with peripheral cells. We demonstrated that exogenous FABP4 interacts with plasma membrane proteins in HUVECs. In addition, we observed that these interactions occurred through specific protein complexes of 77 and 108 kDa, even in FABP4-deficient HUVECs.

This study used two proteomics strategies, both of which identified CK1 as the FABP4-binding protein in the plasma membranes of HUVECs. First, using MALDI TOF/TOF mass spectrometry, we analysed plasma membrane protein lysates from HUVECs incubated with or without FABP4-His after cross-linking and purification with cobalt resin. The MALDI TOF/TOF profiles revealed that CK1 was the putative FABP4-binding protein. We repeated the procedure but used GeLC-MS/MS for the analysis, which also showed that CK1 was the putative receptor. CK1 was again confirmed to be the FABP4 receptor by immunoblotting.

Cytokeratins are known to be part of a family of intermediate filament proteins that participate in cytoskeletal assembly[34]. CK1 is a member of the basic-neutral subfamily of cytokeratins[171]. CK1 consists of a central helical rod domain flanked by 3 loops each consisting of 40 amino acids that protrude from its filament surface. These loops are glycine-rich. CK1 does not have the structure of a transmembrane protein and does not have a phosphatidylinositol linkage. However, the glycine-rich regions contain hydrophobic residues that may loop

through cell membranes and interact with membrane proteins. The glycine loop region is separated from the rod region by the 35 amino acid H1 subdomain, which terminates the penetration of the N-terminal region into cell membranes[172]. In endothelial cells, CK1 appears to play a role as an anchor or receptor for various active molecules[34]. CK1 was also shown to colocalize with urokinase plasminogen activator receptor (uPAR) to form a multiprotein receptor complex for high-molecular-weight kininogen binding at the cell surface[172, 173]. Furthermore, it was demonstrated that endothelial oxidative stress increases CK1 cell-surface protein expression and its ability to bind proteins (mannose-binding lectin)[174] (Fig. 9). In addition, CK1 has been determined to be involved in vascular biology by regulating NO production[34], acting as a scaffolding protein for the assembly of the vasoregulatory plasma kallikrein-kinin system[175]. The kallikrein-kinin system was first recognized as a plasma and tissue proteolytic system that is responsible for the liberation of bradykinin, a vasoactive pro-inflammatory mediator, resulting in NO synthesis and liberation. Tissue plasminogen activator release, superoxide formation, and prostacyclin formation are also induced by bradykinin[34]. It is known that oxidative stress and circulating FABP4 levels are increased in metabolic diseases, such as T2DM and obesity[140-145, 150, 176, 177]. Further studies are needed to confirm that oxidative stress could be involved in FABP4-CK1 interaction. Previous results from our group showed that exogenous FABP4 has a functional role in endothelial cells, inducing endothelial dysfunction. Exogenous FABP4 inhibits the activation of the insulin-signalling pathway, resulting in decreased eNOS activation and NO production[128]. Therefore, from our results, we can speculate that FABP4-CK1 binding could decrease NO production and induce endothelial dysfunction. We demonstrated that pharmacologic FABP4 inhibition decreases complex formation, likely by ameliorating endothelial dysfunction.

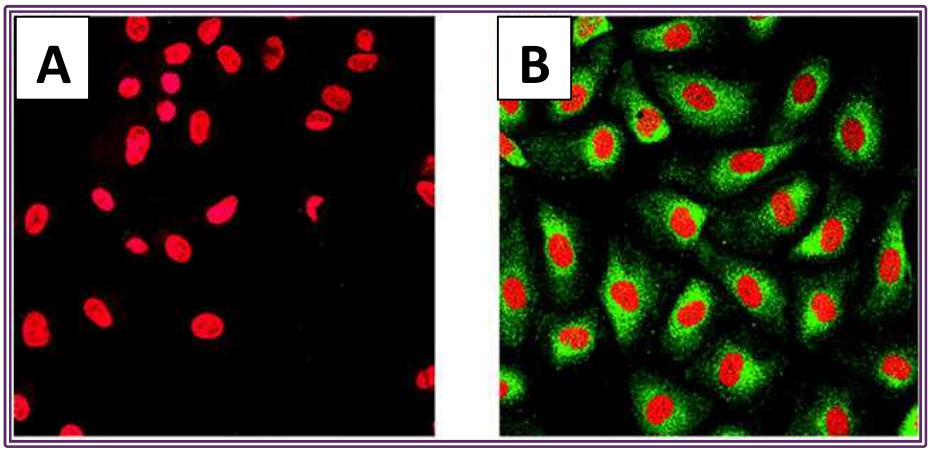

Figure 9. Endothelial CK1 protein (green) expression after oxidative stress was significantly increased (B) compared to normoxic HUVECs (A)[174].

Furthermore, CK1 can be phosphorylated and induce an intracellular signal[34]. Exogenous FABP4 is also able to activate intracellular pathways (e.g., Akt-dependent pathways)[128]; thus, it is possible that this activation also occurs through the FABP4-CK1 interaction.

6.2. Fatty acids are required for the formation of FABP4-CK1 complexes

BMS309403 is a biphenyl azole inhibitor designed to target FABP4. This compound competitively binds within the FABP4 FA-binding pocket and inhibits the binding of FABP4 to endogenous free FAs[170]. We observed that incubation with a FABP4 inhibitor (BMS309403) decreased complex formation between exogenous FABP4 and CK1 in HUVECs. BMS309403 competes with FAs for the FA-binding pocket of FABP4 with high specificity[50], and it is known that FAs produce a ligand-induced

conformational change in FABP4, leading to altered function[127]. Therefore, we suggest that FAs play an important role in the interaction of FABP4 with CK1 in plasma membrane proteins of HUVECs.

Mechanistically, BMS309403 inhibits cholesterol efflux and inflammatory responses in macrophages, suppresses FA uptake in adipocytes in a FABP4-dependent manner and stimulates glucose uptake through selective activation of the AMPK signalling pathway[50, 170]. It is possible that the protection against atherosclerosis and T2DM provided by BMS309403 is due in part to a decrease in FABP4 complex formation. BMS309403 also inhibits lipid accumulation[170, 178]; pathological tissue lipid accumulation was shown to impair insulin signalling and cause IR, which ultimately can develop into T2DM. Several other diseases, such as MS, NAFLD, atherosclerosis, and myocardial infarction, are characterized by pathological lipid deposition within organs. Tight control of lipid uptake and storage in non-adipose tissues is therefore of vital importance. The endothelium represents a novel control point for regulating LCFA uptake, thus limiting excessive tissue lipid accumulation and consequently IR[179]. The importance of the endothelium is known for the insulin-signalling cascade as well as its ability to thereby control muscular glucose[180]. Three groups of proteins have been implicated in LCFA transport into cells: FATPs, scavenger receptor CD36, and FABPs[179, 180]. Changes in FA transporter content or function, as may be caused by nutritional, hormonal, or pharmacological stimuli, will likely have an impact on whole body lipid metabolism[181]. The secretion of FABP4 from adipocytes occurs at baseline conditions and responds to several signals associated with fasting conditions. Additionally in obesity, adipose tissue exhibits signs of inflammation and cell death, which may contribute to the presence of high levels of FABP4 in circulation[147]. Regardless, the apparent role of FABP4 as a required serum component for dysregulated liver glucose production argues that

obesity-induced hyper-FABP4-emia might contribute to elevated hepatic glucose production, which is the hallmark of hyperglycaemia, in subjects with T2DM. This would be consistent with the fact that during the transition from IR to hyperglycaemia, FA concentrations do not change significantly and hence may not alone explain the alterations in liver glucose metabolism[147]. In fact, emerging data have strongly linked serum FABP4 levels with metabolic disease risk in humans and even suggested that circulating FABP4's relation to metabolic risk is significantly stronger than fasting free FAs[147]. These findings also begin to suggest that membrane FA transporters may be exploited as therapeutic targets[181].

6.3. Exogenous FABP4 is internalized into HUVECs

We observed that exogenous FABP4 could be internalized into HUVECs, passing through the cell membrane into the cytoplasm after 30 min and into the nucleus after 2 h. It is known that CK1 is an endothelial receptor that aids in the internalization of myeloperoxidase[175], so it is possible that CK1 also aids in the internalization of FABP4. However, it is not known whether this internalization occurs due to the interaction between FABP4 and CK1 or whether free FABP4 (15 KDa) can pass through the membrane into the cytoplasm; both means of internalization are possible. We do know, however, that FABP4 and CK1 interact only transiently in the plasma membrane given that levels of the protein complex decreased after 5 min. In support of our results, Aragonès et al. has observed that the amount of intracellular FABP4 increases following FABP4 incubation in endothelial cells[128]. In another study, it has also been shown that exogenous FABP4 can be taken up into cells[182].

A previous study from our group demonstrated that exogenous FABP4 induces endothelial cell dysfunction *in vitro,* which was mediated by the interaction of FABP4 with the insulin-signalling pathway in vascular cells. FABP4 alters eNOS activation (through the reduction of eNOS phosphorylation at $Ser^{1177}$and NO production) and reduces insulin receptor substrate 1 (IRS1) phosphorylation at Tyr^{989} and Akt phosphorylation at Ser^{473} [128]. These data suggest that FABP4 has a role in the development of metabolic alterations and its internalization may play an important role in it.

However, we do not know the role of this internalized FABP4. We observed that exogenous FABP4 arrives in the cytoplasm after 30 min, and it is known that FABP4 delivers FAs to cytoplasmic compartments of the cell, such as the mitochondria, for further utilization[109, 183]. In the cytoplasm, FABP4 interacts with HSL, which is an 84-kDa cytoplasmic protein involved in triacylglycerol and cholesterol ester hydrolysis[126]. FABP4 physically associates with HSL in a FA-dependent manner with high affinity and specificity, and its activation by FABP4 is dependent on its ability to bind FAs[126]. Furthermore, HSL is involved in the regulated secretion of FABP4[139]. FABP4 also interacts with unphosphorylated JAK2 (basal state) in a FA-dependent manner and results in attenuation of the JAK2 signalling pathway[28]. JAK2 is part of a family of non-receptor-tyrosine kinases that play a critical role in signal transduction from ligands that bind to members of the cytokine receptor superfamily. Upon ligand binding, one or more of the JAK kinases is activated through transphosphorylation and subsequently phosphorylates the receptor and downstream signalling molecules, such as STAT proteins[28]. The FA dependence of FABP4/JAK2 interaction allows FABP4 to act as a rheostat of intracellular FA levels, affecting metabolism in the cell[28, 103].

Our results also indicate that exogenous FABP4 may be related with gene transcription as it is able to be seen in the nucleus after 2 h. FABP4 has a nuclear localization signal (NLS) and a nuclear export signal (NES)[97] and translocates to the nucleus via passive diffusion[184]; direct ligand binding between FABP4 and FAs enhanced its nuclear localization[112, 184]. A recent study has indicated that FABP4 is shuttled continuously between the nucleus and cytoplasm[103, 184] and that continuous shuttling is involved in transcriptional activation of PPARγ[103, 184] by delivering free FAs to the nucleus, which serve as ligands for PPARs to regulate gene transcription[96, 103, 109, 185]. However, FABP4 markedly enhanced the transcriptional activity of PPARγ both in the absence and in the presence of FA, so there is not a ligand dependency of the FABP4-PPAR interaction[112]. This evidence supports that fact that FABP4 could also act as a transcription factor. Furthermore, in this study we also demonstrated that exogenous FABP4 induced the active forms of the nuclear transcription factors c-jun and c-myc, which are regulated by MAPK cascades in HCASMC even though we do not know if this activation is due to a direct effect of exogenous FABP4 or through the MAPK pathway activation in HCASMCs.

6.4. Exogenous FABP4 forms protein complexes in HAECs, HCASMCs, HepG2 and THP-1 cells

We showed that this interaction did not just occur in HUVEC cells but that it also took place in other cell types, such as HAEC, HCASMC, HepG2 and THP-1 cells. FABP4 is secreted from adipocytes regulated by lipolytic pathways[138, 139] via a non-classical, calcium-dependent mechanism[137]. Although circulating FABP4 has been described as a clinical biomarker of CVD and metabolic diseases[103], these studies suggest that FABP4 is involved in causative links between adiposity and related

CVDs, being also a causative factor for CVD. It is especially striking that exogenous FABP4 was able to form a protein complex with CK1 in HepG2 cells because these cells do not express FABP4, supporting the importance of the interaction of circulating FABP4 with peripheral tissues.

The liver is the major site of LDL biogenesis and cholesterol synthesis as well as the site of BA synthesis. FA oxidative pathways are also active in the liver[88]. Only a single FABP is expressed at a high level in the liver, FABP1[88], which is unique among the FABP family in its ability to bind more than one LCFA/LCFA-CoA and in the size of the ligand that it can accommodate[186]. It also binds other lipids, such as intermediates in LCFA anabolism and catabolism, a variety of steroid-like ligands in FA metabolism and storage and ligands involved in lipid signalling, growth regulation and fat storage, that impact the development of obesity[186]. In obesity, hepatic glucose production is dysregulated and represents a key process leading to development of T2DM[147]. It was demonstrated that secreted FABP4 from adipocytes directly increases hepatic glucose output in mice[139, 147], and *in vitro* studies showed direct actions of FABP4 in liver cells in the regulation of glucose production, which stimulates hepatic gluconeogenesis[147]. Furthermore, the common characteristic of obesity and T2DM is a decrease in insulin action on target tissues, defined as IR[187]. ER stress is a key player in the progression of IR because it could transduce some effects of lipid metabolites and cytokines into an activation of stress kinases that disrupts insulin signalling[187]. FABP4 has been associated with IR[80, 103] and ER stress[105, 131, 188]. In this study, we observed that exogenous FABP4 interacts with CK1 in the plasma membrane of HepG2 cells, demonstrating a direct interaction between secreted FABP4 and hepatic cells, which suggests that FABP4 has a role linking adiposity and hepatic IR and ER stress, although we do not know

if this causative link is due to a direct effect of FABP4 on hepatic cells or to FABP4-CK1 interactions.

The formation of lesions of atherosclerosis takes place in the endothelium, and these lesions increase macrophage recruitment and the formation of foam cells. Furthermore, after intimal injury, mediator-release induces phenotype changes of VSMCs that can migrate and proliferate from media to the intima[189]. Our results show that exogenous FABP4 is able to interact with endothelial cells, VSMCs and macrophages, forming protein complexes with CK1 in the plasma membrane.

FABP4 expression was recently discovered in vascular endothelial cells and, although its role in adipocytes and macrophages has been extensively investigated, its function in endothelial cells is not completely known[93, 108]. FABP4 promotes the proliferation of endothelial cells and has a role in the activation of several mitogenic pathways and expression of several key mediators of angiogenesis[93, 108, 190]. FABP4 plays a proangiogenic role in endothelial cells by promoting cell proliferation, migration, survival, lipid accumulation, and morphogenesis[93, 108, 190]. The interaction of circulating FABP4 with endothelial cells through CK1 may be involved in the angiogenesis process, and it is known that atherosclerotic lesions are associated with angiogenesis within the vessel wall[191].

In addition to endothelial cells, VSMCs represent one of the major cell types of the vascular wall and contribute to vessel wall inflammation and lipoprotein retention, as well as to the formation of the fibrous cap that provides stability to the plaque in atherosclerosis[36]. Plaque ruptures may also heal as a result of proliferation and matrix deposition by VSMCs together with the regression of inflammation[158]. It is known that PVAT-derived factors, such as adipokines, cytokines, and growth factors, may directly target VSMCs (modulating vasculature function)[192]; it is also known that secretory FABP4 promotes the growth and migration of HASMCs *in*

vitro[193]. In this study, apart from demonstrating that exogenous FABP4 interacts with HCASMCs, we also demonstrated that it is involved in migration and proliferation.

In atherosclerosis, FABP4 is mainly colocalized to areas with inflammatory cell infiltrates[158]. Macrophage infiltration is one of the major risk factors for developing symptomatic atherosclerotic lesions[158]. Atorvastatin, a cholesterol-lowering medication, prevents the increase in the expression of FABP4 caused by oxLDL in macrophages[102], which suggests that FABP4 may be involved in foam cell formation. Moreover, it was demonstrated that FABP4 gene expression accelerates cholesterol and triglyceride accumulation in macrophage foam cells, affecting the expression of some key genes for lipid metabolism[194]. In this study, we demonstrated that exogenous FABP4 interacts with the plasma membrane of THP-1 monocyte-derived macrophages through CK1 and that this interaction may be involved in foam cell formation and atherogenesis. It is also known that the expression of pro-inflammatory cytokines was reduced in macrophages from *Fabp4*$^{-}$/$^{-}$ mice when exposed to modified lipoproteins[123] and in macrophages incubated with the pharmacological FABP4 inhibitor (BMS309403)[104]. We demonstrated that exogenous FABP4 can modulate protein expression (migration- and proliferation-related proteins in HCASMCs), even though we do not know how this modulation is produced; thus, circulating FABP4 may be responsible for foam cell formation and pro-inflammatory cytokine production.

These observations, along with the fact that exogenous FABP4 forms a complex with CK1 in endothelial cells (HAEC and HUVECs), VSMCs (HCASMCs) and THP-1 monocyte-derived macrophages, support the notion that circulating FABP4 significantly contributes to pathogenesis of atherosclerosis and other vascular injuries, as it connects vascular and cellular lipid accumulation to inflammation.

FABP4 inhibition could be an effective strategy to improve endothelial dysfunction and to reduce VSMC migration and proliferation, plaque formation and inflammation associated with atherosclerosis.

6.5. Exogenous FABP4 affects migration and proliferation in HCASMCs through ERK1/2 and PI3K activation

Previous results of this study showed that exogenous FABP4 is able to form a specific complex with CK1, suggesting that it could have an effect on peripheral cells, so we analysed the cellular effect of exogenous FABP4 on HCASMCs. Our results show that exogenous FABP4 induces dose-dependent HCASMC proliferation and that the effect is specific because the addition of an anti-FABP4 antibody inhibits the cellular proliferation. In addition to cell proliferation, we found that FABP4 also increases cell migration in a dose- and time-dependent manner. These results are in agreement with a recent study showing that recombinant and secretory FABP4 causes an enhancement of growth and migration in human aortic smooth muscle cells through ROS-mediated mechanisms[193]. The induction of cultured HCASMC migration and proliferation by FABP4 suggests a potential role for this adipokine in promoting vascular pathology. FABP4 exhibits biological activity in various cell types to promote a pro-inflammatory state and vascular dysfunction[185]; however, nothing was previously known about the direct influence of FABP4 on HCASMC activation and the intracellular signalling pathways involved.

We studied different mechanisms for FABP4-induced HCASMC proliferation and migration. The involvement of the ERK1/2 MAPK cascade and the PI3K/Akt signalling pathway in VSMC proliferation and migration has previously been demonstrated[39, 195, 196]. The c-Raf/MEK/ERK pathway is essential for cell

proliferation and migration[197], and our results indicate that FABP4 increases the phosphorylation of ERK1/2, which suggests that FABP4 utilizes the ERK pathway to induce VSMC proliferation and migration. We found that although FABP4 had no effect on Akt activation, an inhibitor of PI3K (LY294002) blocked the effects of FABP4 on HCASMC proliferation and migration, which suggests the activation of an additional kinase pathway. Crosstalk between the Ras/c-Raf/MEK/ERK pathway and other signalling pathways appears to occur. Moreover, the selective inhibition of MAPK kinase by PD98059 significantly reduced FABP4-induced HCASMC proliferation and migration. Taken together, these data clearly demonstrate that FABP4-induced HCASMC proliferation and migration are dependent on ERK1/2 and PI3K activation without the direct involvement of Akt activation.

The translocation of phosphorylated ERK to the nucleus can activate transcription factors, such as c-myc and c-jun, which was demonstrated in the present study. Thus, this suggests that the induction of the c-myc and c-jun transcription factors was initiated by FABP4 through the activation of the c-Raf/MEK/ERK pathway. Although the observed increases in the levels of these transcription factors were not striking, the addition of specific inhibitors of c-jun (SP600123) and c-myc (10074G5) significantly attenuated FABP4-induced HCASMC proliferation and migration. It has been widely observed that modest changes in transcription factor levels can have a great impact on the concentrations of regulated proteins. Cyclin D1 and matrix metalloproteinase-2 (MMP2), CCL2 (or MCP-1), and fibulins 4 and 5 are downstream genes of c-fos and c-myc transcription factors and are involved in cell cycle regulation and cell migration, respectively. We found that the treatment of HCASMCs with FABP4 increased cyclin D1 expression and cell proliferation through c-myc, as demonstrated by the addition of inhibitors of c-jun and c-myc. This effect was more striking when we evaluated the expression of genes involved

in cell migration (MMP2, CCL2, fibulins 4 and 5). Thus, it is conceivable that increases in MAPK signalling by extracellular FABP4 lead to increased levels of cell migration-related proteins rather than increased levels of proteins related to cell proliferation.

We have observed that the amount of intracellular FABP4 increases after FABP4 incubation, suggesting that FABP4 could be internalized by HCASMCs, as in HUVECs. Despite this, we do not know whether the FABP4 effects on VSMC proliferation and migration are due to its interaction on the plasma membrane with CK1 or because it internalizes into the cells. Both means of action are possible. It is known that CK1 can be phosphorylated[34], supporting the fact that exogenous FABP4 can activate metabolic pathways through CK1 interactions. Moreover, in HUVECs, we observed that exogenous FABP4 crosses the plasma membrane to the cytoplasm and the nucleus. These data suggest that FABP4 may have similar behaviour in HCASMCs, with the internalized FABP4 being responsible for ERK1/2 and PI3K pathway activation.

6.6. *Mechanisms of action proposed*

Given these results, we propose a mechanism of action of circulating FABP4 in endothelial cells and VSMCs (Fig. 10).

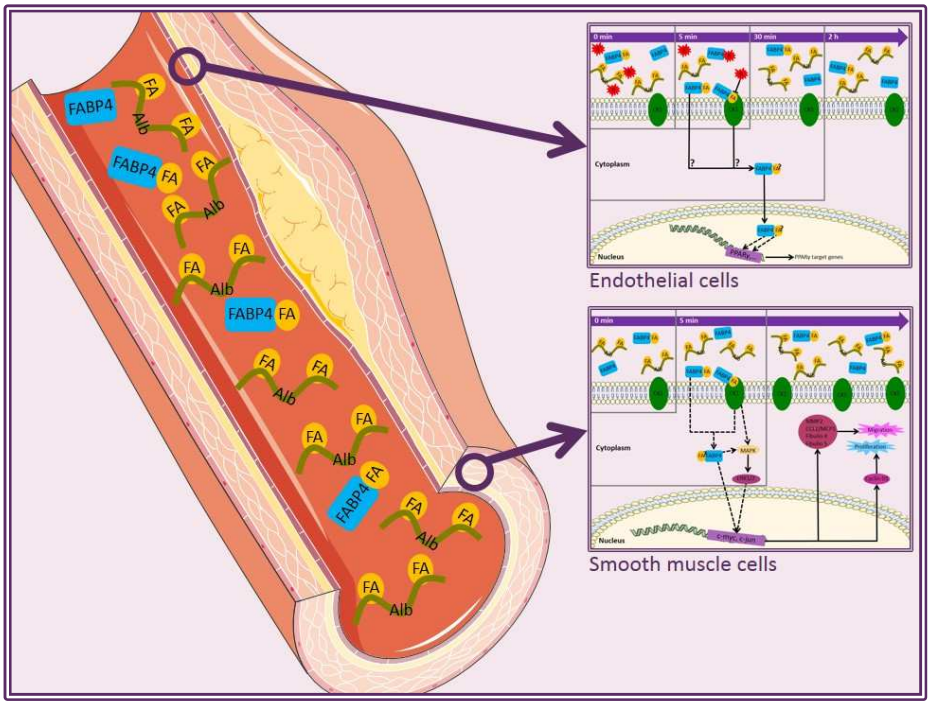

Figure 10. Presence of FABP4 in atherosclerosis and the mechanisms of actions proposed. Abbreviations: Alb, Albumin; FA, Fatty Acid; FABP4, Fatty acid-binding protein 4.

In endothelial cells (Fig. 11), circulating FABP4 is able to interact with the plasma membrane. This interaction requires FAs. When FABP4 is bound to FAs, it presents the active conformation, which is able to interact with CK1 to form a specific protein complex after 5 min. After FABP4-CK1 interaction, the presence of FABP4 in the plasma membrane starts decreasing and FABP4 crosses the plasma membrane, arriving in the cytoplasm after 30 min. We do not know if this internalization is due

to its interaction with CK1 or to passive diffusion through the plasma membrane, and we do not know whether the internalized FABP4 is bound to an FA. Through passive diffusion, circulating FABP4 arrives in the nucleus after 2 h, where it may act as a transcription factor. It is known that FABP4 activates the transcription of PPARγ by itself or by delivering FAs.

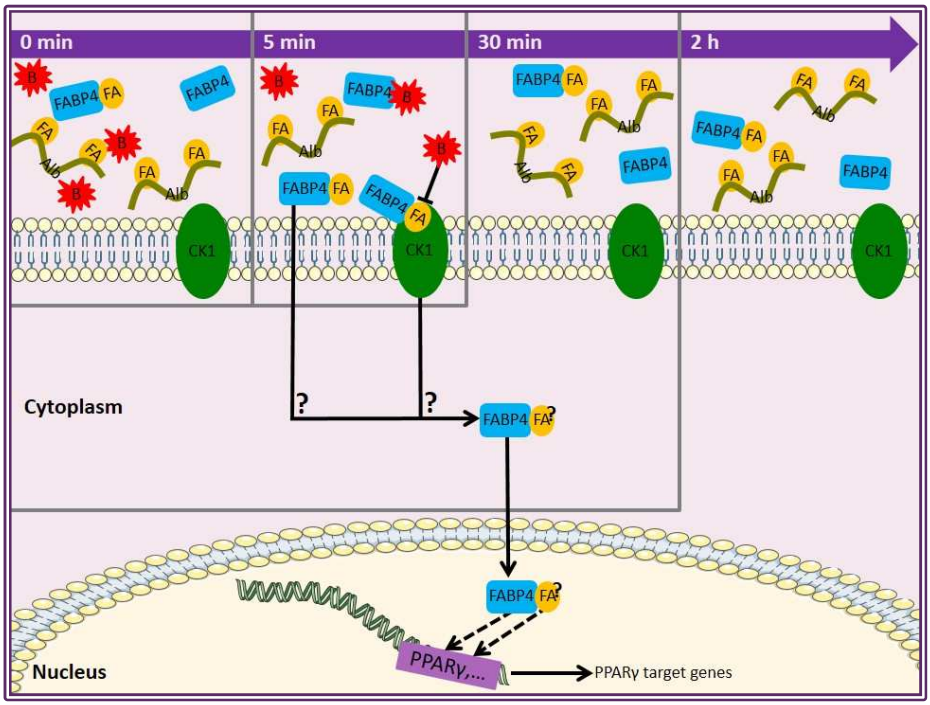

Figure 11. Mechanism of action proposed of circulating FABP4 in endothelial cells. Abbreviations: Alb, Albumin; B, BMS309403; CK1, Cytokeratin 1;FA, Fatty Acid; FABP4, Fatty acid-binding protein 4; PPARγ, Peroxisome proliferator activated receptor γ.

In VSMCs (Fig. 12), circulating FABP4 is involved in migration and proliferation. We observe that circulating FABP4 is also able to form a protein complex with CK1 on the plasma membrane at 5 min. After 5 min of FABP4 incubation, MAPK pathway is activated through phosphorylation of ERK1/2, probably due to the interaction of

FABP4 with CK1. Furthermore, intracellular levels of FABP4 are increased after 5 min of incubation with exogenous FABP4, and those high levels are maintained for 15 min, suggesting that exogenous FABP4 internalization having a direct effect on the activation of MAPK pathway could be another option. Later, the transcription factors c-jun and c-myc in VSMCs are activated, probably through a MAPK-dependent pathway, but it is possible that circulating FABP4 arrives in the nucleus to act as a transcription factor by itself or by delivering FAs.

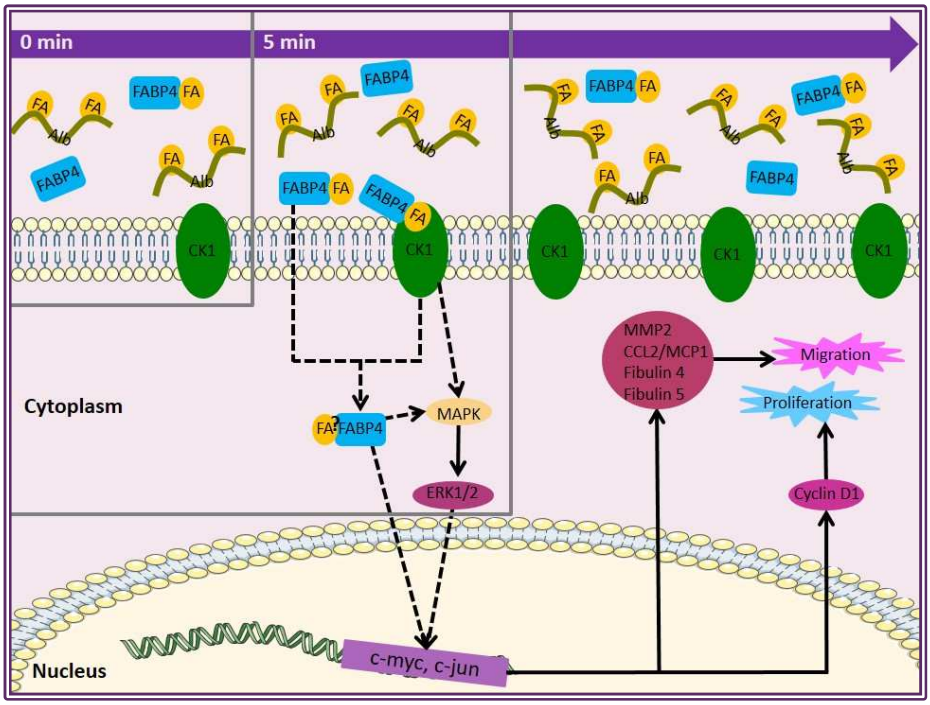

Figure 12. Mechanism of action proposed of circulating FABP4 in VSMCs.
Abbreviations: Alb, Albumin; CCL2/MCP1, monocyte chemoattractant protein-1; CK1, Cytokeratin 1; ERK1/2, Extracellular signal-regulated kinase 1/2; FA, Fatty Acids; FABP4, Fatty acid-binding protein 4; MAPK, Mitogen activated protein kinase; MMP2, Matrix metalloproteinase-2.

7. Conclusions

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- FABP4 is not just a biomarker of metabolic diseases. FABP4 is an adipokine as it is produced and secreted by adipose tissue and interacts with other peripheral cells.

- Exogenous FABP4 is able to interact with plasma membrane proteins, forming specific protein complexes with CK1 in HUVECs and other peripheral cells.

- The presence of BMS309403 decreases the protein complex formation between exogenous FABP4 and CK1, so FAs are required to form FABP4 protein complexes. Therefore, pharmacological inhibition of FABP4 can modulate its mechanism of action by reducing activity associated with the FABP4-CK1 interaction.

- FABP4 is able to cross the plasma membrane into the cytoplasm and reach the nucleus, although its role in those locations is not fully known.

- FABP4 directly promotes the proliferation and migration *in vitro* of HCASMCs through the activation of the ERK1/2 MAPK signalling pathway, activating the nuclear transcription factors c-myc and c-jun and that increases the expression of their downstream genes cyclin

D1 and MMP2, CCL2, and fibulins 4 and 5, involved in cell cycle regulation and cell migration, respectively.

- FABP4 represents a potential therapeutic target for the prevention of CVDs that are associated with obesity. FABP4 inhibition should be explored as a potential therapeutic strategy for treating atherosclerosis and reducing cardiovascular risk.

8. References

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9. Annex I

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ORIGINAL

Interacción de FABP4 con proteínas de membrana de células endoteliales☆

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PALABRAS CLAVE
FABP4;
Células endoteliales;
Membrana celular;
Receptor

Resumen

Introducción: Fatty acid-binding protein 4 (FABP4) es una adipocina secretada por el tejido adiposo implicada en la regulación del metabolismo energético y la inflamación. FABP4 circulante se asocia con obesidad, dislipidemia aterogénica y síndrome metabólico. Estudios recientes muestran una asociación entre FABP4 circulante y disfunción endotelial, aunque se desconoce cómo se produce esta. El objetivo de este trabajo es estudiar la interacción entre FABP4 con las proteínas de la membrana citoplasmática en células endoteliales.

Metodología: Se incubaron células HUVEC con y sin FABP4 (100 ng/ml) durante 5 min. La inmunolocalización de FABP4 se estudió mediante microscopia confocal. Para estudiar las interacciones de FABP4 con las proteínas de membrana de las células HUVEC se diseñó una estrategia que combina incubaciones con o sin 6XHistidine-tag FABP4 (FABP4-His) (100 ng/ml), *cross-linking* con formaldehído, extracción de proteínas de membrana y western blot.

Resultados: Los resultados muestran que FABP4 colocaliza con CD31, una proteína utilizada como marcador de membrana citoplasmática. Además se observan diferentes patrones de western blot en función de la incubación con o sin FABP4-His. El inmunoblot revela la existencia de 3 complejos proteicos de aproximadamente 108, 77 y 33 kDa formados por FABP4 exógena y su posible receptor/es.

Discusión: Los resultados obtenidos apoyan la existencia de un complejo proteico capaz de unir FABP4 a las células endoteliales mediante una unión específica. Además, nos permiten avanzar en el conocimiento de los efectos moleculares de FABP4 y, en caso de confirmarse, podrían utilizarse como diana terapéutica para prevenir enfermedades cardiovasculares.

☆ Una comunicación referente a esta línea de trabajo, titulada «Interacción de FABP4 con proteínas de membrana en células endoteliales», fue presentada en el XXV Congreso Nacional de la SEA-Reus 2012 y galardonada con una mención especial.

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KEYWORDS
FABP4;
Endothelial cells;
Plasma membrane;
Receptor

Interaction of FABP4 with plasma membrane proteins of endothelial cells

Abstract
Introduction: Fatty acid binding protein (FABP4) is an adipose tissue-secreted adipokine implicated in the regulation of the energetic metabolism and inflammation. High levels of circulating FABP4 have been described in people with obesity, atherogenic dyslipidemia, diabetes and metabolic syndrome. Recent studies have demonstrated that FABP4 could have a direct effect on peripheral tissues and, specifically, on vascular function. It is still unknown how the interaction between FABP4 and the endothelial cells is produced to prompt these effects on vascular function. The objective of this work is studying the interaction between FABP4 and the plasma membrane proteins of endothelial cells.
Methodology: HUVEC cells were incubated with and without FABP4 (100 ng/ml) for 5 minutes. Immunolocalization of FABP4 was studied by confocal microscopy. The results showed that FABP4 colocalizates with CD31, a membrane protein marker.

A strategy which combines 6XHistidine-tag FABP4 (FABP4-His), incubations with or without FABP4-His (100 ng/ml), formaldehyde cross-linking, cellular membrane protein extraction and western blot, was designed to study the FABP4 interactions with membrane proteins of HUVECs.
Results: The results showed different western blot profiles depending of the incubation with or without FABP4-His. The immunoblot revelead three covalent protein complexes of about 108, 77 and 33 kDa containing FAPB4 and its putative receptor.
Discussion: The existence of a specific binding protein complex able to bind FABP4 to endothelial cells is supported by these results. The obtained results will permit us advance in the molecular knowledge of FABP4 effects as well as use this protein and its receptor as therapeutic target to prevent cardiovascular.

Introducción

La *fatty acid-binding protein 4* (FABP4; adipocyte-FABP; aP2) es un miembro de la familia de proteínas intracelulares que se unen a ácidos grasos (FABP)[1]. Las FABP pertenecen a una familia multigénica con al menos 9 tipos distintos de proteínas que codifican para proteínas de ∼15 kDa formadas por 126-134 aminoácidos, y que tienen una expresión específica de tejido[1,2].

La estructura génica de la familia de las FABP está altamente conservada entre los miembros estudiados. Además, esta conservación también se mantiene con otros miembros de la familia multigénica de proteínas que se unen a lípidos. Las FABP se expresan abundantemente (1-5% proteínas citosólicas) en células involucradas activamente en el metabolismo lipídico. Los miembros de esta familia presentan un patrón de expresión tejido-específica y se nombran según el tejido en el que fueron identificados por primera vez[3].

Las FABP son capaces de unir una gran variedad de ligandos hidrofóbicos, como por ejemplo ácidos grasos de cadena larga, eicosanoides, leucotrienos y prostaglandinas[4-6]. Sin embargo, la secuencia divergente entre los miembros de esta familia confiere a las proteínas pequeñas diferencias en cuanto a las propiedades de unión a ligandos y también define diferentes patrones de interacción proteína-proteína dependiendo del contexto celular. Se ha visto que las FABP adipocitaria, epitelial y cardiaca interaccionan con la lipasa sensible a hormonas, mientras que la intestinal y la hepática no interaccionan con ella[3,7]. Se sabe también que la FABP4 interacciona con JAK2 de una manera ácido graso-dependiente, estableciendo un nuevo rol para FABP4 como sensor de ácidos grasos y afectando al metabolismo celular mediante interacciones proteína-proteína[8].

La FABP4 se expresa predominantemente en el tejido adiposo y en macrófagos, y juega un papel importante en el metabolismo lipídico e inflamación de ambos tipos celulares[8]. En adipocitos, la FABP4 interacciona con PTEN, lo que sugiere un nuevo papel para esta fosfatasa en la regulación del metabolismo lipídico y en la diferenciación adipocitaria[9]. En macrófagos, participa en la regulación de la actividad inflamatoria y del tráfico de colesterol vía NF-κB y *peroxisome proliferator-activated receptor* (PPAR)-γ, interacción que podría ser responsable de efectos opuestos en la supervivencia y la proliferación en células endoteliales[10].

Los niveles de FABP4 circulantes están aumentados en la obesidad, el síndrome metabólico (SM)[11], la diabetes mellitus tipo 2 (DM2) y la hiperlipidemia familiar combinada o síndromes lipodistróficos, y se correlacionan con lípidos y resistencia a la insulina[12-18]. Los niveles de FABP4 en suero predicen el riesgo de desarrollar SM, DM2 y enfermedad aterosclerótica[17,19-21]. La FABP4 también tiene un efecto directo en la disminución de la contractilidad de las células musculares de miocardio, lo cual sugiere que la liberación de FABP4 al torrente circulatorio podría tener un efecto directo sobre algunas células y tejidos periféricos[22]. Las FABP4 en lesiones ateroscleróticas están asociadas con un fenotipo de placas inestables[23,24].

Un estudio reciente de nuestro grupo ha demostrado que los niveles de FABP4 circulante están inversamente asociados con la hiperemia reactiva periférica, un marcador de disfunción endotelial[25]. En este sentido, resultados de

nuestro grupo muestran que la FABP4 exógena disminuye la expresión y la activación de eNOS[26]. La FABP4 tiene un efecto directo sobre la migración y la proliferación de células humanas musculares de arteria coronaria lisa a través de la activación de MAPK y los factores de transcripción *c-jun* y *c-myc*[27].

Por todo ello, el objetivo de este trabajo es estudiar la interacción de FABP4 exógena con proteínas de membrana de células endoteliales.

Material y métodos

Reactivos

Las células utilizadas en este estudio fueron *Human umbilical vein endothelial cells* (HUVEC) (GIBCO, Oregon, OR, EE. UU.) en pase 3 (C-015-5C). Las células se sembraron en medio M-200 (Cascade Biologics, New York, NY, EE. UU.) suplementado con 2% Low Serum Growth Supplement (LSGS) (Cascade Biologics) y con 1% Gentamicin/Amphotericin solution (Cascade Biologics) en placas de cultivo y/o portas con cámaras individuales (Nunc, Roskilde, Dinamarca). La FABP4 recombinante humana era de BioVendor (Heidelberg, Alemania) y 6XHistidine-tag FABP4 recombinante humana era de Enzo Life Sciences (San Diego, CA, EE. UU.). El anticuerpo anti-FABP4 era de R&D System (Minneapolis, MN, EE. UU.), el anticuerpo anti-6X His-tag era de Abcam (Cambridge, MA, EE. UU.), y los anticuerpos anti- CD31, anti-mouse, anti-rabbit y anti-goat eran de DAKO (Glostrup, Dinamarca). Los anticuerpos marcados con fluorescencia, Alexa Fluor*488 anti-goat y Alexa Fluor*532 anti-mouse, eran de Invitrogen (Carlsbad, CA, EE. UU.). Dulbecco's phosphate-buffered saline (DPBS) era de GIBCO® (14190-169). Paraformadehído era de Sigma (St. Louis, MO, EE. UU.) (P6148-500G) y Mowiol era de Calbiochem (San Diego, CA, EE. UU.) (475904). Las pastillas anti-proteasas sin EDTA (Complete ULTRA Tablets, Mini EDTA-Free EASYpac) eran de Roche (Indianapolis, IN, EE. UU.). El kit FABP4 Inhibitor/Ligand Screening Assay Kit era de Cayman Chemical Company (Ann Harbor, MI, EE. UU.), y el kit HisPur™ Cobalt Purification Kit era de Pierce (Rockford, IL, EE. UU.).

Cultivos celulares y reactivos

Las células HUVEC crecieron con medio 2% LSGS durante 15 min y se incubaron con y sin FABP4 (100 ng/ml) a diferentes tiempos. Tras la incubación en placas de Petri de 10 cm se procedió a la extracción de proteínas de membrana. Se eliminó el medio de cultivo y las células se lavaron con DPBS, seguidamente se añadió el tampón de extracción (50 mM Tris-HCl pH = 7,4, 150 mM NaCl, 2 mM Ca^{2+}, 1% TX100, 1% NP40, antiproteasas)[28]. Se recogieron las células en un tubo, se rompieron pasándolas a través de una aguja (5/8 in) y se incubaron 10 min en hielo. Finalmente se centrifugan a 14.000 xg durante 10 min y se recupera el sobrenadante.

Inmunoblot

La concentración total de proteína se cuantificó con el método Bradford (Bio-Rad). La electroforesis y el inmunoblot se realizaron utilizando el sistema de análisis NuPAGE (Invitrogen Life Technologies, Reino Unido). Las membranas fueron bloqueadas con reactivo ECL Advance Blocking Reagent al 2% (Amersham Biosciences, EE. UU.) e incubadas con anticuerpos anti-FABP4, anti-6X His-tag y anti-CD31. Los complejos antígeno-anticuerpo fueron detectados incubando la membrana con anticuerpos anti-IgG conjugados con HRP. Las bandas se visualizaron con los reactivos ECL (Amersham Pharmacia, EE. UU.) en el sistema de imagen ChemiDoc y se cuantificaron con el programa de análisis Image Lab (Bio Rad, EE. UU.). Los niveles relativos de FABP4 se cuantificaron y se normalizaron con los niveles de CD31; todos los valores se expresaron en unidades arbitrarias.

Inmunofluorescencia

Después de la incubación con y sin FABP4, las células fueron lavadas una vez con solución de lavado (PBS + 2% FBS + 0,1% BSA) durante 5 min, fijadas con paraformaldehído 4% durante 20 min a 4 °C y lavadas con solución de lavado 3 veces, 5 min. La incubación con los anticuerpos primarios anti-FABP4 y anti-CD31 (proteína utilizada como marcador de membrana citoplasmática) se llevó a cabo durante toda la noche a 4 °C. Tras los lavados, las células fueron incubadas

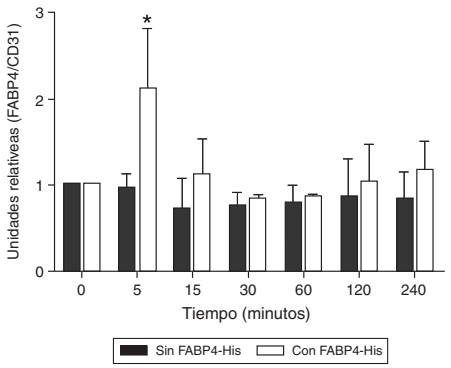

Figura 1 Variación de la cantidad de FABP4 en la membrana citoplasmática de HUVEC en función de la incubación con y sin FABP4 y del tiempo. A) Western blots de lisados de proteínas de membrana de células HUVEC después de la incubación con y sin FABP4 exógena a diferentes tiempos, usando anticuerpos anti-FABP4 y anti-CD31. CD31 se utiliza para corregir el error de carga. B) Gráfico de barras de la comparación cuantitativa entre los niveles de FABP4 en membrana citoplasmática. La intensidad de la señal de las bandas fue analizada por el programa ImageLab a partir de imágenes de 3 experimentos independientes. Los valores de FABP4 fueron normalizados utilizando los valores de CD31 y se expresan como niveles de proteína relativos en cada tiempo. Estos resultados son expresados como media ± error estándar. *$p < 0,05$ cantidad de FABP4 en la membrana de las células incubadas con FABP4 exógena durante 5 min versus cantidad de FABP4 en la membrana de las células incubadas sin FABP4 exógena durante 5 min y versus cantidad de FABP4 en la membrana de las células incubadas con FABP4 exógena a 30 min, 1 y 2 h.

con los anticuerpos secundarios Alexa Fluor*488 anti-goat y Alexa Fluor*532 anti-mouse y lavadas 3 veces con la solución de lavado. Los portas se montaron con mowiol con *antifading* y las imágenes de microscopia confocal de inmunofluorescencia fueron tomadas con Nikon Eclipse TE2000-E y procesadas con el programa EZ-C1 3.40.

Las imágenes de inmunofluorescencia también fueron captadas con un microscopio invertido Olympus IX71, procesadas con el programa CellF y cuantificadas con el programa ImageJ.

Cross-linking en HUVEC

Tras la incubación con y sin FABP4 exógena se realizó el *cross-linking* en las células HUVEC con formaldehído al 2% en DPBS a temperatura ambiente durante 30 min. La reacción se paró con 5 ml de glicina 0,125 M[29,30]. Tras esto, las células se lavaron con DPBS y se extrajeron las proteínas de membrana citoplasmática.

Purificación proteica a partir de cola de histidina

La FABP4-His-tag del extracto de las proteínas de membranas de las células HUVEC fue purificada del resto de proteínas a partir de la cola de histidina. La purificación se hizo siguiendo las instrucciones de *HisPurTM Cobalt Purification Kit* (Pierce).

Ensayos de unión a ligando

Concentraciones crecientes de proteína (de 0 a 60.000 pg/ml) de membrana de células HUVEC incubadas 5 min con y sin FABP4 exógena fueron analizadas con el kit *FABP4 Inhibitor/Ligand Screening Assay Kit* (Cayman) siguiendo las instrucciones del mismo. Brevemente, este kit detecta ligandos de FABP4. Los extractos de proteínas de membrana se incubaron con una sonda que muestra fluorescencia creciente cuando se une a FABP4. La presencia en la muestra de proteínas que se unen a FABP4 desplaza la sonda fluorescente y la fluorescencia emitida decae. Se utiliza ácido araquidónico como control positivo y FABP4 recombinante a una concentración de 10 mM. La pérdida de fluorescencia se monitoriza excitando a 475 nm y leyendo la emisión a 370 nm en un fluorímetro Synergy HT (BioTek, Winooski, VT, EE. UU.). Los resultados obtenidos se presentan como porcentaje de fluorescencia de cada muestra respecto al máximo de fluorescencia (FABP4 + sonda fluorescente).

Análisis estadístico

Los resultados se presentan como media ± error estándar. Los resultados representan la media de 3 experimentos independientes cada uno realizado por duplicado. Los análisis estadísticos se realizaron utilizando el programa Graphpad

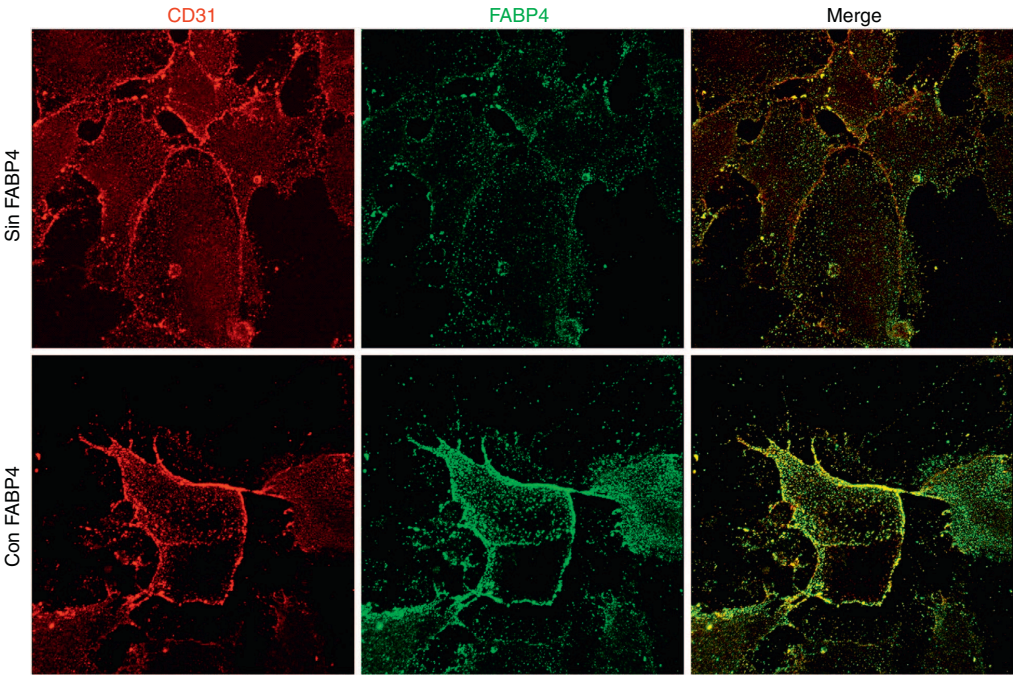

Figura 2 Colocalización de FABP4 exógena en la membrana de células endoteliales. Imágenes de microscopia confocal de una doble inmunofluorescencia para CD31 (rojo) y FABP4 (verde) realizada en 2 condiciones: incubación con FABP4 exógena e incubación sin FABP4 exógena.

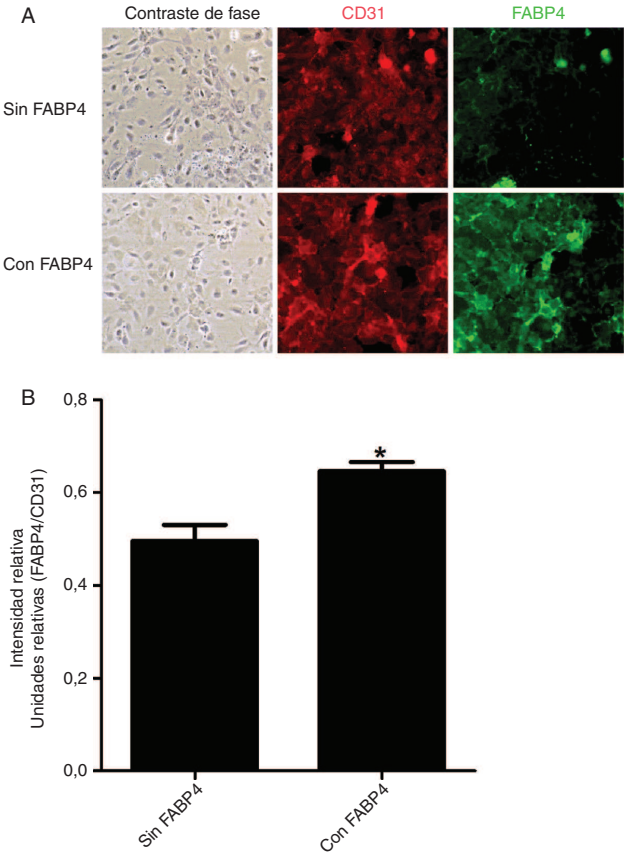

Figura 3 Imágenes y cuantificación de fluorescencia en HUVEC en función de la incubación con y sin FABP4. A) Doble inmunofluorescencia para CD31 (rojo) y FABP4 (verde) realizada en 2 condiciones: incubación con FABP4 exógena e incubación sin FABP4 exógena. Imágenes de fluorescencia obtenidas mediante un microscopio invertido. B) Gráfico de barras que muestra la cuantificación de la inmunofluorescencia de FABP4 tras la normalización con los valores de CD31.*$p < 0,05$ cantidad de FABP4 en la membrana de las células incubadas con FABP4 exógena versus cantidad de FABP4 en la membrana de las células incubadas sin FABP4 exógena.

Prism (versión 5, San Diego, CA, EE. UU.). Se utilizó el test t-Student para comparar medias. Las diferencias entre medias se consideraron estadísticamente significativas con un valor de $p < 0,05$.

Resultados

FABP4 colocaliza con CD31 en membrana de células HUVEC

Tras la incubación de las células HUVEC con FABP4-His a diferentes tiempos (5 min, 15 min, 30 min, 1 h, 2 h y 4 h) se observó un aumento significativo ($p < 0,05$) en los niveles de FABP4 en los extractos de proteínas de membrana a los 5 min (2,22; $p < 0,05$), volviendo a los niveles basales tras ese tiempo. Cuando las células no estaban incubadas con FABP4 exógena, los niveles de FABP4 permanecían constantes durante las 4 h (fig. 1).

Los experimentos de inmunofluorescencia mostraron colocalización de FABP4 con CD31 (proteína utilizada como marcador de membrana citoplasmática) en la membrana de las células endoteliales (fig. 2) tras 5 min de incubación con FABP4 exógena. En la figura 3 observamos que la inmunocolocalización de FABP4 con CD31 se acompaña de un aumento significativo en los niveles de fluorescencia de FABP4 ($p < 0,05$).

FABP4 interacciona específicamente con proteínas de membrana plasmática en células HUVEC

Realizamos un ensayo de unión a ligando *(FABP4 Inhibitor/Ligand Screening Assay Kit)* utilizando proteínas de membrana de HUVEC incubadas con y sin FABP4 exógena durante 5 min. Observamos un aumento en la fluorescencia cuando las células eran incubadas con FABP4 exógena

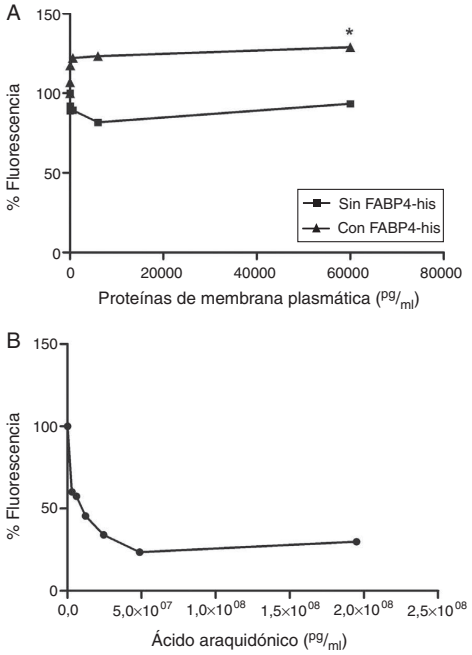

Figura 4 Curvas de desplazamiento del ensayo de unión a ligando. A) Comparación de las curvas de desplazamiento de las proteínas de membrana citoplasmática de células HUVEC incubadas con y sin FABP4 exógena. B) Curva de desplazamiento del ácido araquidónico, control positivo del kit.*$p < 0,05$ diferencia en el porcentaje de fluorescencia entre las proteínas de las células incubadas con FABP4 y las incubadas sin FABP4 exógena a partir de una concentración de 60 pg/ml.

(fig. 4A), sugiriendo la presencia de FABP4 exógena en la membrana. También observamos que cuando usábamos las proteínas de membrana de las células que habían sido incubadas sin FABP4 exógena, la señal de fluorescencia disminuía sugiriendo la presencia de proteínas de membrana capaces de unirse a FABP4 (fig. 4A). A partir de la concentración proteica de 60 pg/ml, las diferencias entre la fluorescencia de las proteínas de membrana de las células incubadas con y sin FABP4 exógena son significativas ($p < 0,05$). El ácido araquidónico fue utilizado como control positivo (fig. 4B).

La figura 5A muestra diferentes patrones de bandas de inmunoblot en función de la incubación con o sin FABP4-His. En el control negativo no se detectó ninguna banda, mientras que en las células incubadas con FABP4-His se detectaron 4 bandas diferentes: 15, 33, 77 y 108 kDa aproximadamente. En el control de especificidad (células HUVEC incubadas con FABP4 a 100 ng/ml y con FABP4-His a 10 ng/ml) se detectaban únicamente las bandas de 15 y 33 kDa indicando que los complejos de 77 y 108 kDa eran complejos específicos formados por FABP4 exógena y otras proteínas. Cuando revelamos estas muestras con anticuerpo anti-6X His-tag (fig. 5B), encontramos el mismo patrón de bandas que observábamos con anti-FABP4.

Discusión

El principal resultado de nuestro trabajo es la existencia de proteínas de membrana de células endoteliales capaces de formar complejos proteicos específicos con FABP4 exógena.

En estudios previos se ha postulado FABP4 como biomarcador plasmático del SM, DM2 y enfermedad aterosclerótica[17,19-21]. Además, el aumento de las concentraciones de FABP4 plasmática se considera un riesgo para los tejidos periféricos, ya que modula la vía de la insulina en células vasculares y, consecuentemente, disminuye la activación de eNOS y la producción de NO, afectando a la vasodilatación arterial. Estos resultados apoyan el papel directo que juega FABP4 en la disfunción endotelial[26]. Resultados previos de nuestro grupo muestran que FABP4 circulante también tiene un efecto directo en las complicaciones observadas en la lesión vascular en DT2[25].

En este trabajo se demuestra la interacción entre FABP4 circulante y la membrana citoplasmática de las células HUVEC (figs. 2 y 3), lo cual apoya la hipótesis de que la FABP4 circulante no es solamente un biomarcador sino que puede que tenga un efecto causal directo al interaccionar con células periféricas. Además, observamos que esta interacción se produce a través de la formación complejos proteicos específicos (fig. 4). A partir de estos resultados, nuestro grupo está trabajando en la identificación de las proteínas que se unen a FABP4 para formar estos complejos, lo que sería necesario para conocer el papel de la FABP4 plasmática en estas enfermedades y entender mejor la función biológica de esta proteína. Se sabe que la FABP cardiaca (62% de homología con FABP4) tiene la capacidad de unirse a un receptor de membrana descrito en células cardiacas, observación que no ha sido confirmada ni demostrada en otras FABP[31].

La FABP4 intracelular interacciona con el receptor de insulina, y los ácidos grasos modifican la estructura de la FABP4 activándola como sustrato del receptor de insulina[32]. No se sabe si esta interacción se produce forma directa o si es a través de otros componentes de la membrana plasmática. Además, la FABP4 endógena es una diana de la ruta VEGF/VEGFR2 y un regulador positivo de la proliferación de células endoteliales in vitro[10].

No se sabe todavía si la FABP4 circulante puede internalizarse y activar mecanismos intracelulares. Se ha visto que la cantidad de FABP4 intracelular en células endoteliales aumenta cuándo las células se incuban con FABP4 exógena en comparación con células no tratadas, lo que puede ser un indicio de la internalización de FABP4 circulante. Además, este aumento en la concentración intracelular de FABP4 va acompañado de una inhibición en la producción de eNOS y NO, debido a una alteración en la ruta de eNOS mediado por insulina a causa de la inhibición de la activación de IRS1 y Akt[26]. Se sabe también que la FABP4 exógena regula la proliferación en células musculares a través de la activación de MAPK y los factores de transcripción c-jun y c-myc[27].

Todos estos datos sobre la interacción y la activación de rutas de la FABP4 exógena apoyan la hipótesis de su internalización. Además es probable que, una vez internalizada, la FABP4 pueda llegar hasta el núcleo, en donde active factores de transcripción o incluso actúe como factor de transcripción.

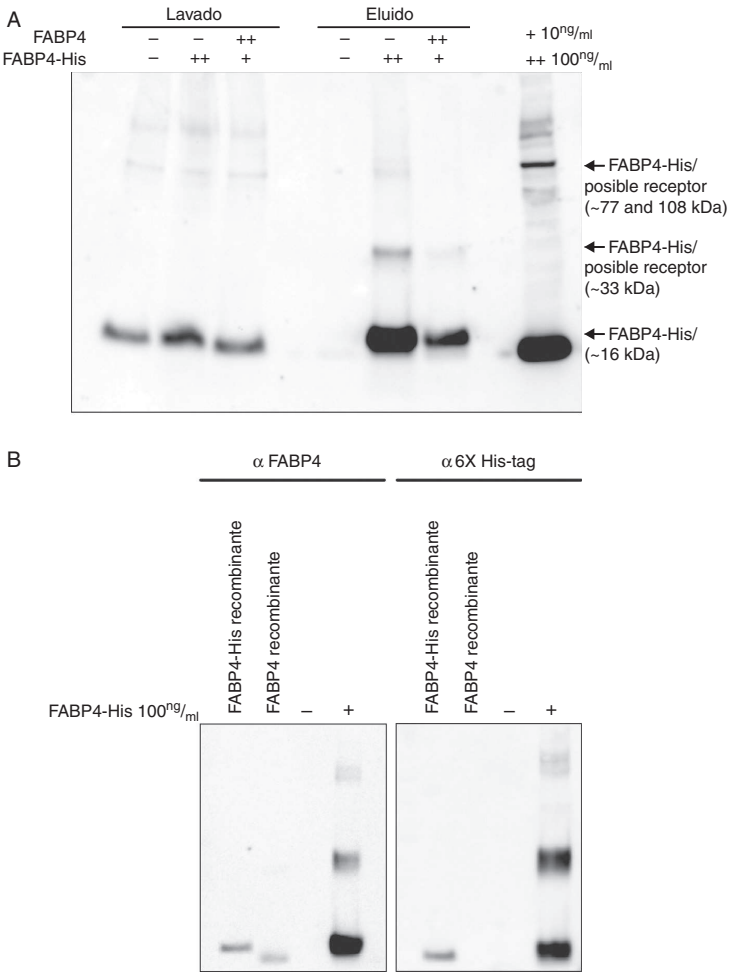

Figura 5 Formación de complejos en la membrana citoplasmástica de HUVEC con FABP4-His. A) Western blot de extractos de proteínas de membrana citoplasmática de células HUVEC después de la incubación con y sin FABP4-His y después de la purificación a partir de la cola de histidina, utilizando anticuerpos anti-FABP4. B) Western blot de extractos de proteínas de membrana citoplasmática de células HUVEC después de la incubación con y sin FABP4-His y después de la purificación a partir de la cola de histidina, utilizando anticuerpos anti-FABP4 y anti-6X His-tag.

En conclusión, estos resultados muestran por primera vez la existencia de un complejo proteico específico que se forma a partir de la unión de FABP4 exógena con las células HUVEC. Los resultados obtenidos nos permiten avanzar en el conocimiento de los efectos moleculares de la FABP4, pudiéndose utilizar como diana terapéutica para prevenir enfermedades cardiovasculares asociadas con la obesidad y la diabetes.

Responsabilidades éticas:

Protección de personas y animales. Los autores declaran que para esta investigación no se han realizado experimentos en seres humanos ni en animales.

Confidencialidad de los datos. Los autores declaran que en este artículo no aparecen datos de pacientes.

Derecho a la privacidad y consentimiento informado. Los autores declaran que en este artículo no aparecen datos de pacientes.

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Autoría

Todos los autores han contribuido en el diseño y análisis de los datos, así como en la redacción del artículo. La versión final de este manuscrito está aprobada por todos los autores.

Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

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10. Annex II

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Abbreviations:

ABCA1	ATP-Binding Cassette A1
ACC	Acetyl-CoA Carboxylase
Akt	Protein Kinase B
AMPK	AMP-activated Protein Kinase
AP-1	Activator Protein 1
Apo	Apolipoprotein
ATGL	Adipose Triglyceride Lipase
BA	Biliary Acid
BMI	Body Mass Index
CK1	Cytokeratin 1
CRP	C-reactive Protein
CVD	Cardiovascular Disease
DPP4	Dipeptidyl Peptidase-4
eNOS	Endothelial Nitric Oxid Synthase
ER	Endoplasmic Reticulum
ERK	Extracellular Signal–regulated Kinase
FA	Fatty Acid
FABP	Fatty Acid Binding Protein
FOXO1	Forkhead Box Protein O1
HCASMC	Human Coronary Artery Smooth Muscle Cell
HSL	Hormone-sensitive Lipase
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Intercellular Adhesion Molecule
IGF-1	Insulin-like Growth Factor-1
IL	Interleukin
iLBP	Intracellular Lipid-binding Proteins
IR	Insulin Resistance
JAK2	Janus Kinase 2
JNK	c-Jun N Terminal Kinase
LCFA	Long Chain Fatty Acid
LDL	Low-density Lipoprotein
MAPK	Mitogen-activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1

MS	Metabolis Syndrome
NAFLD	Non-alcoholic Fatty Liver Disease
NF-κB	Nuclear Factor kappa B
NK cells	Natural Killer cells
NO	Nitric Oxide
NQO1	NAD(P)H:quinine Oxidoreductase 1
Nrf2	Nuclear Factor Erythroid 2 (NFE2)-related Factor 2
PAI-1	Plasminogen Activator Inhibitor-1
PDGF	Platelet Derived Growth Factor
PI3K	Phosphatidylinositol 3-kinase
PPAR	Peroxisome Proliferator-activated Receptor
PTEN	Phosphatase and Tensin Homolog Deleted on Chromosome 10
PVAT	Perivascular Adipose Tissue
RBP4	Retinol Binding Protein 4
ROS	Reactive Oxygen Species
T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumor Necrosis Factor α
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VSMC	Vascular Smooth Muscle Cell
ΔDCN	Decorin

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